ANALYSIS OF THE ROLE OF THE TRANSCRIPTION FACTOR-C/EBPβ IN IMPLANTATION

BY

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DISSERTATION

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ABSTRACT

During early pregnancy, the concerted actions of the maternal steroid hormones, estrogen and progesterone, promote a unique process known as decidualization, which involves extensive proliferation and differentiation of uterine stromal cells. The molecular pathways underlying this hormonally induced cellular transformation, an essential prerequisite for embryo implantation, are poorly understood. We previously identified the transcription factor C/EBPβ as a target of steroid hormone regulation in the uterus. The uteri of C/EBPβ–null mice failed to undergo decidualization, indicating a critical role of this transcription factor in this process. In the present study, analyses of C/EBPβ-null uteri revealed that C/EBPβ mediates the effects of steroid hormones during decidualization in the mouse by modulating the expression of multiple key cell cycle regulatory factors that control the G2-M transition of the proliferating uterine stromal cells. Utilizing a unique primary human endometrial stromal cell (HESC) culture system, in which the cells undergo proliferation and differentiation in response to serum and steroid hormone and cAMP signaling, respectively, my research uncovered that C/EBPβ controls the G1/S transition of the proliferating HESCs by regulating the expression of specific cell cycle regulators such as cyclin E and E2F1. My studies, using a siRNA strategy, revealed that C/EBPβ also plays an essential role in human stromal differentiation. We employed microarray analysis to establish that several factors, such as BMP2, Wnt4, IL11Ra, and STAT3, are downstream targets of C/EBPβ during differentiation of HESCs. Further analysis of IL11Ra-STAT3 signaling and identification of downstream targets of STAT3 suggested that this signal transducer is a critical mediator of the function of C/EBPβ, providing a novel link between C/EBPβ and cytokine signaling pathways that regulate endometrial functions during decidualization. The knowledge

gained from this study provides a better understanding of the molecular mechanisms underlying the decidualization phase of implantation. This research also will help us decipher the underlying causes of infertility and recurrent loss of early pregnancy.

To Father and Mother

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CHAPTER 1

Introduction

1.1 Introduction

Steroid hormones estrogen and progesterone regulate uterine functions during early pregnancy

The physiological functions of mammalian uterus are profoundly influenced by the concerted and timely actions of the ovarian steroid hormones, estrogen (E) and progesterone (P). During pregnancy, these hormones orchestrate the changes in the uterine epithelium that makes it competent to attach to the embryo to initiate the process of implantation (1-4). Following the initial attachment step, the trophoblast invades the endometrial stroma, which then undergoes extensive proliferation, differentiation and remodeling, a process known as decidualization. This leads to the formation of "decidua", a unique and transient maternal tissue that helps maintain an environment conducive for the growth and development of the implanting embryo until the placenta is formed. Although it is well-documented that uterine competence for embryo implantation, and the formation and function of the decidua are greatly influenced by E and P, the molecular pathways involved in this hormonally-induced cellular transformation event remain poorly understood. Furthermore, it is becoming increasingly evident that perturbations in these pathways may lead to improper uterine receptivity, decidualization, and eventual infertility due to early pregnancy loss. There is, therefore, a need to explore these hormone-regulated pathways to gain a better understanding of the dysfunctions that result in female infertility.

Although the details of the events leading to implantation vary in different species, the central roles played by E and P in controlling various phases of early pregnancy are common to many mammals (5). In mice, on days 1 and 2 of pregnancy, ovarian E released at ovulation induces luminal and glandular epithelial cell proliferation. On day 3, the rising level of progesterone from the newly formed corpora lutea shifts the

proliferation from the epithelium to the stroma and switches the uterine epithelium to differentiate and become receptive. A transient surge of E on day 4 of pregnancy triggers the implantation process (5). Then a generalized uterine edema promotes the closure of the uterine lumen and facilitate close attachment between the trophectoderm of the blastocyst and the uterineepithelium (6).

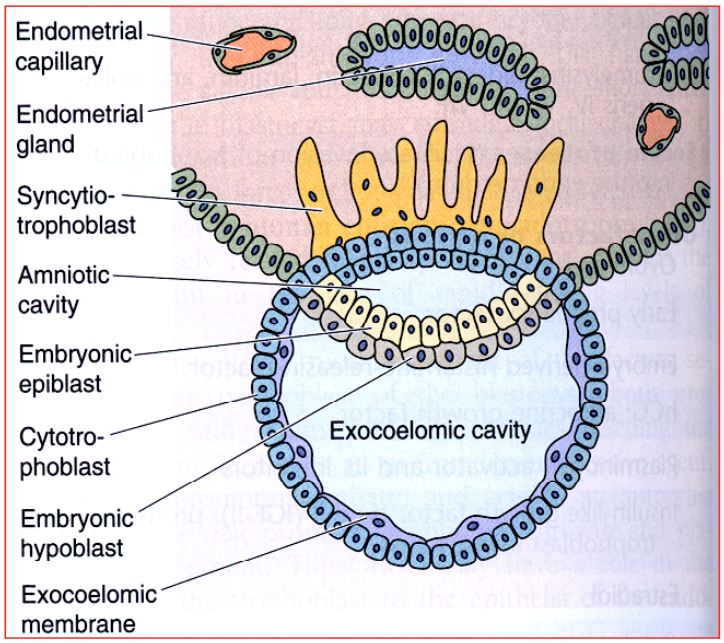

Figure 1.1 Trophoblast invasion into the uterine stroma during implantation.

(Adapted from Medical Physiology by Walter Boron and Emile Boulpaep)

In mice, the blastocyst reaches the uterus four days after fertilization (7;8). The attachment of the blastocyst to the uterine epithelium initiates the process of decidualization that involves differentiation of the fibroblastic endometrial stromal cells into morphologically distinct cells, termed decidual cells (9-14). These transformed cells show unique secretory and biosynthetic properties. This cellular differentiation, induced by P following a brief priming by E, is a prerequisite for successful implantation. Decidua is a transient tissue, which first develops at the time of blastocyst attachment on day 4.5 of pregnancy. During the next 3 days of gestation, the decidual cells proliferate and differentiate extensively, and then they start to undergo apoptosis. By the end of the invasive period (day 10.5), the decidua is regressed. A variety of functions have been

attributed to the decidua, such as providing nutrition to the embryo, being a source of hormones, growth factors and cytokines, serving an immunoregulatory role during pregnancy and regulating trophoblast invasion (9-14). Steroid-induced signaling molecules that participate in the formation and function of this tissue remain poorly understood.

Role of E- and P- regulated pathways in implantation

The mouse has served as an important animal model to study the regulation of uterine functions by E and P (5;15-17). The cellular actions of these hormones are mediated through intracellular estrogen receptor (ER) and progesterone receptor (PR) proteins, which are hormone-inducible transcription factors (18). Hormone-occupied ER or PR triggers the expression of specific gene networks in different cell types within the uterus and the products of these genes are thought to mediate the hormonal effects during the reproductive cycle and pregnancy. The heterogeneous cell types (luminal and glandular epithelium, stroma and myometrium) in the uterus respond differently to E and P. In general, estrogen induces the proliferation of luminal and glandular epithelial cells, while progesterone stimulates the proliferation and differentiation of stromal cells and maintains the pregnancy.

The development of mutant mouse models lacking the estrogen receptor α (ERα) and progesterone receptor (PR) has firmly established the requirement of these hormones and their downstream signaling pathways for successful establishment and maintenance of pregnancy (15;16). The ERα-null mice are impaired in uterine growth and preparedness for blastocyst attachment (15). The PR-null mice display a refractory uterus that fails to respond to a deciduogenic stimulus (16). A number of studies employing gene expression profiling have explored steroid-regulated pathways that control epithelial and stromal functions during implantation in mice (19-22). Although these studies have identified several potential target genes for both ER and PR, the precise mechanisms by which these factors mediate hormone-regulated functions in the uterus remain unclear.

Identification of C/EBPβ as a novel target of steroid regulation in pregnant uterus

Gene expression profiling in pregnant uterus identified steroid-regulated gene networks that have functional relevance in implantation (23;24). Our studies identified CCAAT/Enhancer Binding Protein beta (C/EBPβ) as a novel mediator of the biological actions of E and P in mouse uterus (23;24). This transcription factor belongs to a family of basic leucine zipper (bZIP) proteins, which regulate numerous biological processes, including cell proliferation, differentiation, metabolic homeostasis, acute phase inflammation and apoptosis (25-27). The C/EBP family members regulate transcription of target genes by binding to a consensus nucleotide sequence motif, ATTCGG/CCAAT, which resides in the regulatory regions of these genes. Previous studies indicated that C/EBPβ is a critical regulator of proliferation and/or differentiation in multiple tissues including the liver, adipose tissue, immune system, skin cells and mammary gland (28-36). In the liver and mammary gland, it displays growth-promoting function (31-33). In contrast, it exerts an antiproliferative action in epidermal keratinocytes and contributes to their growth arrest (36). C/EBPβ is, therefore, implicated in both positive and negative control of cell proliferation, depending on the cellular context.

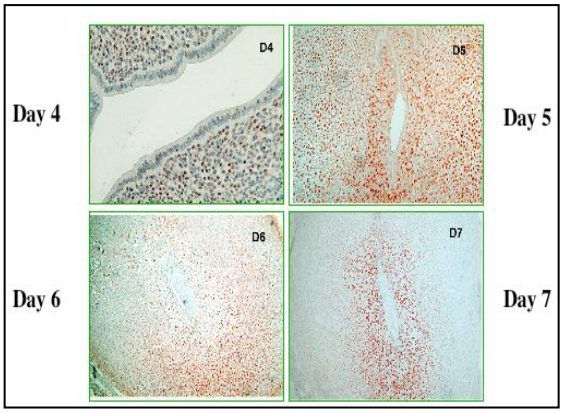

Figure 1.2 Expression of C/EBPβ during normal gestation.

Uterine sections were prepared from tissues collected at days 4-7 of pregnancy and analyzed by IHC using anti-C/EBPb antibody. Panels D4 to D7 represent pregnant uteri at days 4, 5, 6, and 7 of gestation. (Adapted from S. R.. Mantena et al. Proc. Natl. Acad. Sci. USA, 2006.)

Administration of either E or P to ovariectomized non-pregnant mice induced C/EBPβ mRNA and protein in the uterine epithelial as well as stromal cells (23). Since these cells express ER and PR, and the induction by each hormone is lost in the absence of its cognate receptor, it is clear that the expression of this gene is controlled directly or indirectly by these receptors. During normal pregnancy in mice, a markedly enhanced expression of C/EBPβ occurred predominantly in the stromal compartment during the decidualization phase (Fig. 1.2).

Following blastocyst attachment to the epithelium, the underlying stromal cells undergo extensive proliferation and differentiation that result in their transformation into the decidual cells (37-40). In rodents, decidualization can also be experimentally induced by scratching or introduction of a drop of oil in steroid hormone-primed uteri (14;41). We found that the expression of C/EBPβ is robustly induced in the decidual tissue during normal as well as experimentally induced decidual reaction. The decidual stage-specific expression of C/EBPβ likely arises from a complex interplay of E and P within the uterine compartments. Although a transient rise in E in the preimplantation period induces C/EBPβ in the stromal compartment of pregnant uterus, it appears that as these cells become progressively differentiated, PR becomes a critical regulator of this gene. This view is strongly supported by the observation that RU486, an antagonist of PR, efficiently suppressed stromal C/EBPβ expression when administered on day 6 of pregnancy (23).

Role of C/EBPβ in female reproduction: insights from the knockout mouse

Development of a C/EBPβ-null mouse model indicated that female mice lacking C/EBPβ are infertile, while the mutant males are fertile (29). The infertility of C/EBPβ-null females was initially attributed to an impaired corpus luteal function. Consistent with this finding, transplantation of wild-type ovaries into C/EBPβ-null mice restored the formation and function of corpora lutea. This intervention, however, resulted only in a poor rescue of the pregnancy outcome. When normal ovaries were transplanted into C/EBPβ-deficient mice, only rare pregnancies were observed. In contrast, exchange of one ovary of wild-type females with a mutant ovary did not affect pregnancy outcome

and produced about 12-14 pups per litter. These results hinted at additional reproductive abnormalities in the mutant female, presumably at the level of the uterus. Starting from a mixed 129Sv X C57BL/6 genetic background of the C/EBPβ–null mice, we have now established a line with >90% 129Sv background through repeated breeding into this background. In collaboration with the Johnson group, we have identified multiple functional abnormalities in the uterine tissue of the mutant mouse (23;24). Embryos transferred to pseudopregnant uteri of C/EBPβ-null mice failed to implant. The uterine defects in the mutant mice included a reduced epithelial cell proliferation in response to E and a lack of stromal response to a deciduogenic stimulus. These phenotypic defects were observed in the presence of exogenously administered steroid hormones, indicating that they were independent of ovarian malfunction and intrinsic to the uterus.

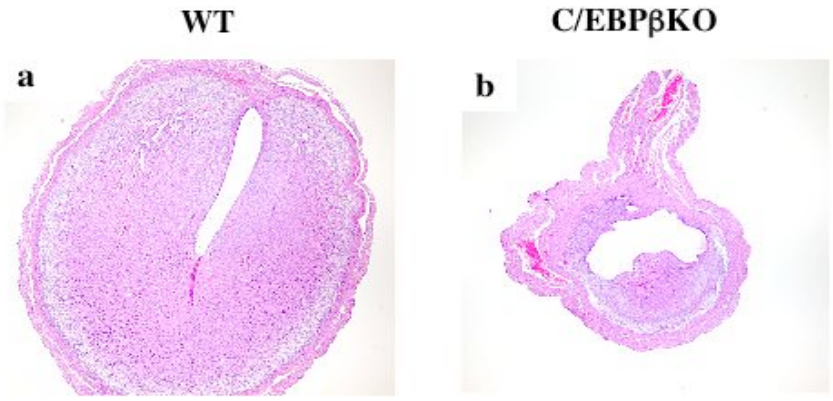

Figure 1.3 C/EBPβ is essential for decidual response.

WT (left panel) and C/EBPβ knockout (KO) (right panel) mice were subjected to artificial decidual stimulation for 72 h. For each mouse, one uterine horn was stimulated, while the other horn was left undisturbed. Histological analysis by hematoxylin and eosin staining of representative uterine sections obtained from a stimulated WT (left panel) and C/EBPβ KO (right panel) mice subjected to artificial decidualization protocol. (Adapted from S. R. Mantena et al. Proc. Natl. Acad. Sci. USA, 2006.)

C/EBPβ regulates stromal cell proliferation during decidualization

During decidualization, the uterine stromal cells undergo proliferation followed by differentiation (37-40) (41). To investigate the decidualization defect in C/EBPβ-null mice, Mantena *et al* monitored the proliferation of steroid hormone-primed stromal cells in response to a mechanical decidual stimulus, using known markers of this event. Uteri of WT mice exhibited an intense staining for Ki67, predominantly in the stromal cells, within 24 h of receiving the decidual stimulus, clearly indicative of extensive stromal cell proliferation, which is also seen during normal pregnancy in response to embryo attachment. In contrast, the uterine sections from C/EBPβ-null mice showed greatly reduced Ki67 staining under identical conditions, implying a defect in stromal cell proliferation in the mutant mouse (23). A major goal of my research is to investigate the molecular basis of the mitotic arrest of the C/EBPβ-null uterine stromal cells. This work is described in Chapter 2.

C/EBPβ is a critical regulator of decidualization in the mouse

The expression of C/EBPβ in the stroma during decidualization prompted our laboratory to analyze the functional role of this transcription factor during this process. The C/EBPβ-null females are infertile (29). To initially examine whether these mutant mice can support pregnancy at all, Mantena *et al* performed embryo-transfer experiments. Blastocysts were collected from uteri of wild-type (WT) females on day 4 of pregnancy. These blastocysts were then transferred directly into the uteri of pseudopregnant WT or C/EBPβ-null females on day 4 of pseudopregnancy. Five days after the embryo transfer, the numbers of implanted embryos as well as the size of the decidua in the recipient uteri were examined. Mantena *et al* found that the majority (>90%) of the transferred embryos did not implant in C/EBPβ-null recipients and a few that were present in the uterus did not grow or form any significant decidua (23). These results indicated that uterine functions are severely compromised in the C/EBPβ-null mice.

Considering the possibility that the impairment in implantation and decidualization in the C/EBPβ-null mice could arise from a potential inadequacy of

ovarian hormone synthesis or release, Mantena *et al* administered steroid hormones to these mice and assessed the response of the hormone-primed uteri to a decidual stimulation in an experimentally induced decidualization experiment. In this protocol, uteri of ovariectomized WT and C/EBPβ-null mice were treated with a well-established regimen of E and P and then decidualization reaction was initiated in the left uterine horn by mechanical stimulation with a blunt needle while the right horn was left unstimulated (13, 41). Mantena *et al* then examined the morphology of the stimulated and unstimulated uterine horns of WT and C/EBPβ-deficient mice (23). As expected, the uterine horn of WT mice exhibited a robust decidual response within 72 h after receiving the mechanical stimulation. In contrast, the C/EBPβ-null uteri under identical conditions failed to show any significant decidualization. Histological examination of WT and null uteri further revealed that the stimulated WT horn was packed with decidual cells (Figure 1.3, left). In contrast, similarly treated uterine horns of C/EBPβ-null animals exhibited a markedly reduced mass of decidual cells (Fig. 1.3, right). Our studies clearly indicated a severe impairment of the decidualization process in the C/EBPβ-null mice and suggested a critical role of this transcription factor in supporting the stromal cell differentiation program in steroid hormone-primed uterus.

The decidualization defect in C/EBPβ-null mice was also confirmed by examining the biochemical markers of this process. Mantena *et al* reported that the expression of alkaline phosphatase, a classical marker of decidualization of stromal cells (42), was prominent in the antimesometrial decidual cells of the WT uteri at 72 h after decidual stimulation. In contrast, no expression of this decidual marker was seen in the uteri of C/EBPβ-null mice at a similar time point, indicating a complete blockade of stromal decidualization. It is important to mention that the stromal expression patterns of PR and ERα in the C/EBPβ-null mice are comparable to those of the WT mice. The loss of stromal cell proliferation and differentiation in the null mice, therefore, does not arise from an aberrant steroid signaling due to a lack of PR and ERa in this compartment, but is rather a direct functional consequence of C/EBPβ-deficiency.

Potential role of C/EBPβ in proliferation and decidualization of human endometrium

During each menstrual cycle, the human endometrium first passes through an E-dominated proliferative phase (d 1-14) involving extensive epithelial regeneration and then enters a P-dominated secretory phase (d 15-28) during which maturation and predecidualization of the stromal compartment occurs in preparation for embryo implantation (43;44). Predecidualization is a process that begins the transformation of stromal cells into decidual cells. It is initiated in stromal cells surrounding the spiral arteries during the mid-late secretory stage of the cycle. If pregnancy ensues, a full-blown decidualization spreads throughout the stroma under the continued influence of E and P. The putative implantation window in the human opens for a short period of time in the mid secretory stage of the cycle (d 20-24)(45). Decidualization of endometrial stroma is essential for successful implantation in the human.

The molecular cues that are known to influence proliferation and decidualization of the human endometrium include steroid hormones E and P, growth factors and cytokines such as leukemia inhibitory factor (LIF), heparin-binding epidermal growth factor (HB-EGF), and interleukin-11, and the regulators of extracellular matrix such as matrix metalloproteases (44;46-50). The identities of the steroid-regulated pathways that control the proliferation and decidualization process are largely unknown. Induction of a few steroid-regulated molecules such as prolactin (PRL), IGFBP-1 and Hoxa10 during decidualization have been described, but their precise functions during decidualization remain unclear (51-54). Our recent studies revealed an intense nuclear expression of C/EBPβ in both epithelial and stromal compartments during proliferative and secretory phases of the menstrual cycle (55). An intense nuclear expression of C/EBPβ in glandular epithelium and differentiating stroma of human endometrium during the mid secretory phase of the cycle (55). These findings raised the possibility that C/EBPβ-regulated pathways control HESC function during various stages of the menstrual cycle.

Extensive earlier studies have shown that endometrial stromal cells isolated from biopsies collected at the proliferative stage of the menstrual cycle can either proliferate or undergo differentiation, depending on the culture conditions (51;56;57). Several

laboratories have shown that the HESCs can be maintained in proliferating state for many generations or induced to undergo differentiation in response to a hormonal cocktail containing P, E, and a cAMP analog (51;56-59). This primary culture system, therefore, presents a unique opportunity to explore the role of C/EBPβ in HESCs proliferation and differentiation. We observed that C/EBPβ expression is markedly induced concomitant with stimulation of HESC proliferation by high serum and the addition of the hormonal cocktail. These finding raised the possibility that this transcription factor is a critical driver of endometrial stromal proliferation and differentiation in the human. A major goal of my research is to exploit this *in vitro* culture system to identify C/EBPβ-regulated downstream pathways during stromal proliferation and differentiation in human. This work is described in Chapter 3 and Chapter 4.

References

1. **Phychoyos A** 1973 Endocrine control of egg implantation. Washington DC: American Physiological Society
2. **Yoshinaga K** 1988 Uterine receptivity for blastocyst implantation. Ann NY Acad Sci, 1988, pp 424-431
3. **Parr MB, Parr EL** 1989 The Implantation Reaction. New York: Plenum Press
4. **Weitlauf HM** 1994 Biology of implantation. New York
5. **Carson DD, Bagchi I, Dey SK, Enders AC, Fazleabas AT, Lessey BA, Yoshinaga K** 2000 Embryo implantation. Developmental biology 223:217-237
6. **Paria BC, Huet-Hudson YM, Dey SK** 1967 Blastocyst's state of activity determines the "window" of implantation in the receptive mouse uterus. Proc Natl Acad Sci, USA, 1967, pp 10159-10162
7. **Psychoyos A** 1986 Uterine receptivity for nidation. Ann N Y Acad Sci 476:36-42
8. **Paria BC, Huet-Hudson YM, Dey SK** 1993 Blastocyst's state of activity determines the "window" of implantation in the receptive mouse uterus. Proc Natl Acad Sci, USA, 1993, pp 10159-10162
9. **DeFeo VJ** 1967 Decidualization. In: Wynn RM ed. Cellular biology of the uterus. Amsterdam: North Holland; 191-290
10. **Finn CA** 1977 The implantation reaction. In: Wynn RM ed. Biology of the Uterus. New York: Plenum Press; 246-308
11. **Glasser SR, Mulholland J, Mani SK, Julian J, Munir I, Lampelo S, Soares MJ** 1991 Blastocyst-endometrial relationships: reciprocal interactions between uterine epithelial and stromal cells and blastocysts. Trophoblast Research 5:229-280
12. **Cross JC, Werb Z, Fisher SJ** 1994 Implantation and the placenta: key pieces of the developmental puzzle. Science 266:1508-1518
13. **Irwin JC, Giudice D** 1999 Encyclopedia of Reproduction. New York: Acadmic Press
14. **Gu Y, Gibori G** 1999 Deciduoma: Academic Press
15. **Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O** 1993 Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. Proc Natl Acad Sci, U S A, 1993, pp 11162-11166
16. **Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery CA, Jr., Shyamala G, Conneely OM, O'Malley BW** 1995 Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. Genes & development 9:2266-2278

17. **Dey SK, Lim H, Das SK, Reese J, Paria BC, Daikoku T, Wang H** 2004 Molecular cues to implantation. Endocr Rev 25:341-373

18. **Tsai MJ, O'Malley BW** 1994 Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Ann Rev Biochem 63:451-486

19. **Cheon YP, Li Q, Xu X, DeMayo FJ, Bagchi IC, Bagchi MK** 2002 A genomic approach to identify novel progesterone receptor regulated pathways in the uterus during implantation. Molecular endocrinology (Baltimore, Md 16:2853-2871

20. **Reese J, Das SK, Paria BC, Lim H, Song H, Matsumoto H, Knudtson KL, DuBois RN, Dey SK** 2001 Global gene expression analysis to identify molecular markers of uterine receptivity and embryo implantation. The Journal of biological chemistry 276:44137-44145

21. **Li Q, Cheon YP, Kannan A, Shanker S, Bagchi IC, Bagchi MK** 2004 A novel pathway involving progesterone receptor, 12/15-lipoxygenase-derived eicosanoids, and peroxisome proliferator-activated receptor gamma regulates implantation in mice. The Journal of biological chemistry 279:11570-11581

22. **Cheon YP, DeMayo FJ, Bagchi MK, Bagchi IC** 2004 Induction of cytotoxic T-lymphocyte antigen-2beta, a cysteine protease inhibitor in decidua: a potential regulator of embryo implantation. The Journal of biological chemistry 279:10357-10363

23. **Mantena SR, Kannan A, Cheon YP, Li Q, Johnson PF, Bagchi IC, Bagchi MK** 2006 C/EBPbeta is a critical mediator of steroid hormone-regulated cell proliferation and differentiation in the uterine epithelium and stroma. Proceedings of the National Academy of Sciences of the United States of America 103:1870-1875

24. **Bagchi MK, Mantena SR, Kannan A, Bagchi IC** 2006 Control of uterine cell proliferation and differentiation by C/EBPbeta: functional implications for establishment of early pregnancy. Cell Cycle 5:922-925

25. **Wedel A, LomsZiegler-heitbrock HW** 1995 The C/EBP family of transcription factors. Immuno Biol 9:171-185

26. **Ramji DP, Foka P** 2002 CCAAT/enhancer-binding proteins: structure, function and regulation. Biochem J 365:561-575

27. **McKnight SL** 2001 McBindall--a better name for CCAAT/enhancer binding proteins? Cell 107:259-261

28. **Tanaka T, Akira S, Yoshida K, Umemoto M, Yoneda Y, Shirafuji N, Fujiwara H, Suematsu S, Yoshida N, Kishimoto T** 1995 Targeted disruption of the NF-IL6 gene discloses its essential role in bacteria killing and tumor cytotoxicity by macrophages. Cell 80:353-361

29. **Sterneck E, Tessarollo L, Johnson PF** 1997 An essential role for C/EBPbeta in female reproduction. Genes & development 11:2153-2162

30. **Tanaka T, Yoshida N, Kishimoto T, Akira S** 1997 Defective adipocyte differentiation in mice lacking the C/EBPβ and /or C/EBPδ gene. EMBO J 16:7432-7443

31. **Greenbaum LE, Li W, Cressman DE, Peng Y, Ciliberto G, Poli V, Taub R** 1998 CCAAT enhancer- binding protein beta is required for normal hepatocyte proliferation in mice after partial hepatectomy. J Clin Invest 102:996-1007

32. **Robinson GW, Johnson PF, Hennighausen L, Sterneck E** 1998 The C/EBPbeta transcription factor regulates epithelial cell proliferation and differentiation in the mammary gland. Genes & development 12:1907-1916

33. **Seagroves TN, Krnacik S, Raught B, Gay J, Burgess-Beusse B, Darlington GJ, Rosen JM** 1998 C/EBPbeta, but not C/EBPalpha, is essential for ductal morphogenesis, lobuloalveolar proliferation, and functional differentiation in the mouse mammary gland. Genes & development 12:1917-1928

34. **Bundy LM, Sealy L** 2003 CCAAT/enhancer binding protein beta (C/EBPbeta)-2 transforms normal mammary epithelial cells and induces epithelial to mesenchymal transition in culture. Oncogene 22:869-883

35. **Tang QQ, Otto TC, Lane MD** 2003 CCAAT/enhancer-binding protein beta is required for mitotic clonal expansion during adipogenesis. Proceedings of the National Academy of Sciences of the United States of America 100:850-855

36. **Zhu S, Yoon K, Sterneck E, Johnson PF, Smart RC** 2002 CCAAT/enhancer binding protein-beta is a mediator of keratinocyte survival and skin tumorigenesis involving oncogenic Ras signaling. Proceedings of the National Academy of Sciences of the United States of America 99:207-212

37. **Das RM, Martin L** 1978 Uterine DNA synthesis and cell proliferation during early decidualization induced by oil in mice. J Reprod Fertil 53:125-128

38. **Moulton BC** 1979 Effect of progesterone on DNA, RNA, and protein synthesis of deciduoma cell fractions separated by velocity sedimentation. Biol Reprod 21:667-672

39. **Moulton BC, Keonig BB** 1984 Uterine deoxyribonucleic acid synthesis during preimplantation procursors of stromal cell differentiation during decidualization. Endocrinology 115:1302-1307

40. **Dey SK** 1996 Implantation. New York: Lipincott-Raven

41. **Glasser SR** 1990 Biochemical and structural changes in uterine endometrial cell types following natural or artificial deciduogenic stimuli. Trophoblast Research 4:377-416

42. **Finn CA, Hinchliffe JR** 1964 Reaction of the mouse uterus during implantation and decidua formation as demostrated by changes in the distribution of alkaline phosphatase. J Reprod Fertil 8:331-338

43. **Bell SC** 1990 Decidualization and relevance to menstruation. Cambridge, U. K.: Cambridge University Press

44. **Giudice LC, Telles TL, Lobo S, Kao L** 2002 The molecular basis for implantation failure in endometriosis: On the road to discovery. Ann NY Acad Sci 955:252-264

45. **Navot D, Scott RT, Droesch K** 1991 The window of embryo transfer and the efficiency of human conception in vitro. Fertil Steril 55:114-118

46. **Mulholland J** 2002 Steroid regulated genes in the endometrium: a reference base. London: Taylor & Francis

47. **Stewart CL** 1994 Leukemia inhibitory factor and the regulation of pre-implantation development of the mammalian embryo. Mol Reprod Dev 39:233-238

48. **Chobotova K, Karpovich N, Carver J, Manek S, Gullick W, Barlow DH, Mardon HJ** 2005 Heparin-binding epidermal growth factor and its receptors mediate decidualization and potentiate survival of human endometrial stromal cells. J Clin Endocrinol Metab 90:913-919

49. **Karpovich N, Klemmt P, Hwang JH, McVeigh JE, Heath JK, Barlow DH, Mardon HJ** 2005 The production of interleukin-11 and decidualization are compromised in endometrial stromal cells derived from patients with infertility. J Clin Endocrinol Metab 90:1607-1612

50. **Osteen KG, Keller NR, Feltus FA, Meiner MH** 1999 Paracrine regulation of matrix metalloproteinase expression in normal human endometrium. Gynecol Obstet Invest 48:2-13

51. **Huang JR, Tseng L, Bischof P, Janne OA** 1987 Regulation of prolactin production by progestin, estrogen, and relaxin in human endometrial stromal cells. Endocrinology 121:2011-2017

52. **Bell SC, Jackson JA, Ashmore J, Zhu HH, Tseng L** 1991 Regulation of insulin-like growth factor-binding protein-1 synthesis and secretion by progestin and relaxin in long term cultures of human endometrial stoma cells. J Clin Endocrinol Metab 72:1014-1024

53. **Giudice LC, Milkowski DA, Lamson G, Rosenfeld RG, Irwin JC** 1991 Insulin-like growth factor-binding proteins in human endometrium: steroid-dependent messenger ribonucleic acid expression and protein synthesis. J Clin Endocrinol Metab 72:779-787

54. **Taylor HS, Bagot C, Kardana A** 1999 HOX gene expression is altered in the endometrium of women with endometriosis. Hum Reprod 14:1328-1331

55. **Plante BJ, Kannan A, Bagchi MK, Yuan L, Young SL** 2009 Cyclic regulation of transcription factor C/EBP beta in human endometrium. Reprod Biol Endocrinol 7:15

56. **Tang B, Guller S, Gurpide E** 1993 Cyclic adenosine 3',5'-monophosphate induces prolactin expression in stromal cells isolated from human proliferative endometrium. Endocrinology 133:2197-2203

57. **Irwin JC, Kirk D, King RJB, Quigly MM, Gwatkin RBL** 1989 Hormonal regulation of human endometrial stromal cells in culture: an in vitro model for decidualization. Fertil Steril 52:761-768

58. **Ryan IP, Schriock ED, Taylor RN** 1994 Isolation, characterization, and comparison of human endometrial and endometriosis cells in vitro. J Clin Endocrinol Metab 78:642-649

59. **Gellersen B, Brosens IA, Brosens JJ** 2007 Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives. Semin Reprod Med 25:445-453

CHAPTER 2

The CCAAT/Enhancer Binding Protein Beta (C/EBPβ) is a Critical Regulator of Steroid-Induced Mitotic Expansion of Uterine Stromal Cells during Decidualization

2.1 Abstract

During early pregnancy, the concerted actions of the maternal steroid hormones, estrogen and progesterone, promote a unique process known as decidualization, which involves extensive proliferation and differentiation of uterine stromal cells. The molecular pathways underlying this hormonally induced cellular transformation, an essential prerequisite for embryo implantation, remain poorly understood. We previously identified the transcription factor C/EBPβ as a target of steroid regulation in the uterus. Uteri of mice lacking C/EBPβ failed to undergo decidualization. In the present study, analyses of C/EBPβ-null uteri indicated that loss of this factor leads to a block in stromal cell proliferation in response to a decidual stimulation. The mutant stromal cells entered S phase of the cell cycle and completed DNA synthesis but were unable to execute mitosis. Further analysis revealed that C/EBPβ facilitates the transition of these cells into mitosis by binding directly to the cyclin B2 promoter to regulate the expression of this cyclin. The expression of cdc25C, a phosphatase that maintains the active state of the cyclin B-cyclin-dependent kinase complex during mitosis, is strongly suppressed in C/EBPβ-null stromal cells, uncovering additional mechanisms by which C/EBPβ controls G2-M transition. Furthermore, the expression of the tumor suppressor p53 and the cell cycle inhibitors p21 and p27 was markedly elevated in C/EBPβ-null stromal cells prior to the mitotic phase. Collectively, these results revealed that C/EBPβ mediates the effects of steroid hormones during decidualization by modulating the expression of multiple key cell cycle regulatory factors that control the G2-M transition of the proliferating uterine stromal cells.

2.2 Introduction

The mouse model has been used extensively to study the molecular signaling mechanisms underlying the process of embryo implantation (1;2). During the preimplantation phase of pregnancy in this species, the maternal steroid hormones, estrogen (E) and progesterone (P), orchestrate molecular and cellular alterations in the uterine surface epithelium that make it competent to attach to the blastocyst to initiate the process of implantation (3-6). The attachment of the blastocyst on day 4.5 of pregnancy triggers the process of decidualization, which involves a remarkable transformation of the fibroblastic endometrial stromal cells underlying the surface epithelium into morphologically and functionally distinct decidual cells (7-12). This cellular transformation process occurs under the influence of E and P during days 5-8 of gestation. Initially the undifferentiated stromal cells undergo mitotic expansion, and then they enter the differentiation program that converts them into decidual cells. The formation of the decidual tissue surrounding the implanting embryo is a prerequisite for successful implantation. It is well documented that the uterine stromal cells acquire unique secretory and biosynthetic activities upon differentiation. They serve as a source of paracrine effectors such as hormones, growth factors and cytokines, which promote uterine angiogenesis and embryo development, mediate immunoregulatory functions during pregnancy, and regulate trophoblast invasion (7-12). An important challenge in the study of implantation biology is to understand the complex process by which steroid hormones regulate the formation and function of the decidual tissue. To this end, it is critical to identify and characterize the factors induced by the maternal hormones that regulate the proliferation and differentiation of uterine stromal cells during the decidualization process.

We previously employed gene expression profiling in pregnant mouse uterus to identify steroid-regulated gene networks that have functional relevance in implantation (13;14). Our studies identified CCAAT/Enhancer Binding Protein beta (C/EBPβ) as a novel mediator of the biological actions of E and P in the uterus during early pregnancy (13;14). This transcription factor belongs to a family of basic leucine zipper (bZIP) proteins, which controls numerous biological processes, including cell proliferation,

differentiation, metabolic homeostasis, acute phase inflammation and apoptosis (15-17). The C/EBP family members regulate transcription of target genes by binding to a consensus nucleotide sequence motif, ATTCGG/CCAAT, which resides in the regulatory regions of these genes. Previous studies revealed that female mice lacking C/EBPβ are infertile, while the mutant males are fertile (18). The infertility of C/EBPβ-null females was initially attributed to impaired corpus luteal function. In subsequent studies, we demonstrated that multiple functional abnormalities in the uterine tissue of the mutant mouse also contribute to the observed infertility (13;14). The uterine defects in the mutant mice included a reduced epithelial cell proliferation in response to E and a lack of stromal response to a deciduogenic stimulus (13). These phenotypic defects were observed in the presence of exogenously administered steroid hormones, indicating that they were independent of ovarian malfunction and intrinsic to the uterus.

During the decidualization phase of pregnancy, the uterine stromal cells undergo proliferation for 24-48 h and then enter the differentiation program (19-22). The lack of decidual response in C/EBPβ-null uteri indicated that this transcription factor is a potential regulator of pathways directing stromal proliferation or differentiation or both. Morphological analysis of uterine sections of wild-type (WT) and C/EBPβ-null uteri in response to decidual stimulus showed a significantly reduced stromal/decidual cell mass in the mutant mice, hinting at a defect in uterine stromal cell proliferation in these mice. In the present study, we tested the hypothesis that C/EBPβ regulates stromal cell proliferation during decidualization by controlling the expression or activity of critical cell cycle regulatory molecules. We performed a detailed analysis of the expression and function of potential cell cycle regulators in the stromal compartment of C/EBPβ-null uteri to pinpoint the molecular defect(s) underlying the lack of decidual response in the mutant tissue.

Our study revealed that, in response to decidual stimulation, C/EBPβ-null uterine stromal cells are able to progress through the G1 and S phases of the cell cycle, but failed to undergo mitosis. The mutant cells displayed markedly reduced levels of cyclin B2 and cdc25C phosphatase, two key regulators of mitosis. We further confirmed that C/EBPβ directly binds to cyclin B2 promoter to regulate its expression. Additionally, we observed

an increased expression of the cell cycle suppressors, p53, p21 and p27, in the mutant cells prior to mitosis. Collectively, these results indicated that C/EBPβ plays a vital role in decidualization by controlling the expression or activity of multiple cell cycle regulatory molecules that critically control G2-M transition.

2.3 Materials and Methods

Animals and Tissue Collection

All experiments involving animals were conducted in accordance with the National Institutes of Health standards for the use and care of animals. The animal protocols were approved by the University of Illinois Institutional Animal Care and Use Committee. Female 129-SV mice (from Charles River, Wilmington, MA) and C/EBPβ-null mice of same genetic background were subjected to experimentally induced decidualization. Mice were euthanized at various time points following the administration of the artificial decidualization stimulus and uteri were collected.

Experimentally induced decidualization

Decidualization was induced artificially as described previously (23). Briefly, mice were subjected to bilateral ovariectomy. Two weeks following ovariectomy, animals were injected with 100 ng of estrogen (Sigma) in 0.10 ml of sesame oil for three consecutive days. This was followed by 2 days of rest, and then administration of daily injections of 1 mg of progesterone (Sigma) and 10ng estrogen for three consecutive days. Six hours after the third P and E injection, 20μl sesame oil was injected into uterine horn to mimic the decidualization stimulus.

Isolation of primary stromal cells and analysis of decidualization in vitro:

Mouse uteri were collected at various time points following artificial decidualization and uterine stromal cell were isolated as described previously (24). Briefly, uterine horns were dissected and cut into 3-5 mm pieces. After washing with Hank's balanced salt solution (HBSS, Invitrogen), uterine tissues were placed in HBSS containing 6 g/liter dispase (Invitrogen) and 25g/liter pancreatin (Sigma) for 1 h at room

temperature and then 10 min at 37 °C. The tissues were gently mixed and the supernatant was discarded to remove the endometrial epithelial clumps. The partially digested tissues were washed twice in HBSS and then placed in HBSS containing 0.5g/liter collagenase (Sigma). After incubation for 45 min at 37 °C, the tubes were vortexed for 10-12 s until the supernatant became turbid with dispersed endometrial stromal cells. The contents of the tube were then passed through a 70-μm gauze filter (Millipore). The stromal cells in the filtrate were pelleted, re-suspended in Dulbecco's modified Eagle's Medium-F12 medium (DMEM-F12 supplemented with 100 unit/liter penicillin, 0.1 g/liter streptomycin, 1.25 mg/liter fungizone, Invitrogen) containing 5% fetal calf serum (Atlanta Biological), and plated. The unattached cells were removed by washing with HBSS, and cell culture was continued after addition of fresh medium supplemented with P (1 μM) and E (10 nM) as described previously (24).

Immunohistochemistry and Immunocytochemistry

Paraffin-embedded endometrial sections were subjected to immunohistochemistry as described previously (25). Polyclonal antibodies against BrdU and phospho-histone 3 (Santa Cruz Biotechnology) were used for immunohistochemistry at dilutions of 1:500 and 1:1000, respectively. Mouse primary stromal cell were fixed and subjected to immunocytochemistry using antibodies against cyclin B2, Cdk1, cdc25C and phospho-Cdk1 (Thr14/Tyr15) (Santa Cruz Biotechnology).

Western Blotting:

Stromal cell were isolated from uteri collected from mice at different times after artificial decidual stimulus as described previously (26). The cells were lysed in ice-cold lysis buffer containing 25 mM Tris/HCl, pH 7.4, 50 mM NaF, 100 mM NaCl, 1 mM sodium vanadate, 5 mM EGTA, 1 mM EDTA, 1% (v/v) Triton X-100, 10 mM sodium pyrophosphate, 1 mM benzamidine, 0.1 mM PMSF, 0.27 M sucrose, 2 μM microcystin and 0.1% (v/v) 2-mercaptoethanol. The cell debris was removed by centrifugation and the protein concentration of the lysate was determined by the method of Bradford, using BSA as a standard. The cell lysates were analyzed by western blotting using antibodies against

cyclin B2, cyclin A, Cdk1 (Santa Cruz Biotechnology), and p53, p21, p27 (Cell Signaling Technology).

Chromatin immunoprecipitation (ChIP) analysis.

The ChIP analysis was performed using the EZ ChIP (Upstate Biotechnology), according to the manufacturer's protocol. Briefly, mouse uterine stromal cell were isolated from wild type mice subjected to artificial decidualization protocol and cultured in the presence of E and P for 21 h. The cells ($8X10^6$) were then placed in PBS buffer and cross-linked with 1% formaldehyde for 10 min. The cross-linked cells were harvested, lysed using SDS lysis buffer, and sonicated. After pre-clearing the lysates with salmon sperm DNA–protein A at 4 °C for 2 h, the DNA–protein complexes in the supernatant were immunoprecipitated using antibodies against RNA polymerase II, mouse IgG (Upstate Biotechnology) or C/EBPβ (Santa Cruz Biotechnology). The immune complexes were recovered by adding protein A agarose. The beads then were washed repeatedly and the bound complexes were eluted using the elution buffer. The cross-linking was reversed and then proteins were digested using 0.5 mg/ml proteinase K. Purified DNA were used as templates for PCR using various primer sets to amplify specific regions of the cyclin B2 promoter.

Adenovirus-mediated expression of dominant negative C/EBPβ

An adenoviral vector expressing a dominant negative mutant of C/EBPβ (A-C/EBP) was provided by Dr. Charles Vinson (NCI/NIH). This vector expresses A-C/EBP under the control of CMV promoter. The protein is expressed with a HA epitope tag. An adenoviral vector lacking the A-C/EBP insert and expressing GFP was used as a control. Stromal cells were isolated from WT uteri collected immediately after artificial decidual stimulation, and allowed to attach to slides for 2 h. Then the cells were cultured in medium supplemented with P (1 μM) and E (10 nM) and transduced with adenovirus expressing dominant-negative A-C/EBP or control virus (Ad-GFP) (MOI is 10:1) or no virus treated. After 20 h transduction, Stromal cells were treated with formaldehyde and ChIP assays were performed as described above.

Statistical Analysis

All experiments were repeated at least three times. For preparation of RNA and protein samples, the uteri were pooled from at least three mice subjected to the same experimental treatment. The real-time PCR results are expressed as mean ± S.D of three separate measurements. Statistical significance of the data was determined using one-way ANOVA followed by student t-test. A p-value of <0.05 was considered significant.

2.4 Results

C/EBPβ-null uterine stromal cells undergo DNA replication in response to a decidual stimulation

To investigate the role of C/EBPβ in the proliferation of steroid hormone-primed uterine stromal cells, we subjected WT and C/EBPβ-null mice to experimentally induced decidualization. Non-pregnant, cycling WT or C/EBPβ-null mice were ovariectomized, rested for two weeks, and treated with a regimen of E and P as per standard protocol (13). Uterine horns of these animals were then subjected to a decidual stimulation as described in the Materials and Methods. To assess DNA synthesis, we monitored the incorporation of bromodeoxyuridine (BrdU) in uterine tissue at different times following decidual stimulation. Previous studies in our laboratory indicated a marked increase in BrdU incorporation in WT uterine stroma at 12 h following decidual stimulation (Wei Wang, and M. K. Bagchi, unpublished observation). Therefore, to examine the S phase activity in the stromal tissue, we administered a two-hour pulse of BrdU at 12 and 14 h following the decidual stimulation and collected uteri at 14 and 16 h, respectively. In the WT animals, a significant portion of the uterine stroma, particularly the sub-luminal stromal cells, exhibited BrdU immunostaining by 14 h (Fig. 2.1 A, left, top panel). By 16 h, strong and widespread BrdU incorporation was seen in the stromal compartment of WT uteri (Fig. 2.1 A, right, top panel). Sections of C/EBPβ-null uteri collected at the same time points following decidual stimulation exhibited similar robust incorporation of BrdU (Fig. 2.1 A, bottom panels). Consistent with these results, quantitation of the BrdU immunostaining revealed no significant difference in the number of BrdU-positive cells

in the stromal compartments of the WT and C/EBPβ-null uteri (Fig. 2.1B). The stromal cells of C/EBPβ-null uteri, therefore, were able to progress through the G1-S checkpoint, enter the S phase and execute DNA synthesis.

C/EBPβ-null uterine stromal cells exhibit impaired mitosis

To monitor the mitotic activity of these mutant stromal cells, we employed an antibody, which recognizes phosphorylated Ser 10 of histone 3 (P-His3), a unique mitotic phase marker (27). In animal cells, the Ser 10 phosphorylation of H3 begins in pericentromeric heterochromatin in late G2 interphase cells. As mitosis progresses, the P-His3 mark spreads throughout the condensing chromatin, and this modification is complete in most cells by the time prophase chromosomes are formed (27). We observed that by 20 h following decidual stimulation, a large number of the stromal cells in the WT uteri displayed specific staining for P-His3, suggesting that they undergo mitotic division (Fig. 2A, upper panel). In contrast, markedly reduced and only sporadic staining for P-His3 was seen in the stromal compartment of C/EBPβ-null uteri at a similar time point, indicating that the majority of the mutant cells are unable to proceed through mitosis (Fig. 2.2 A, lower panel). Quantitation of the immunofluorescence signal indicated an approximately 5-fold decrease in P-His3 staining in C/EBPβ-null stromal cells relative to the WT cells (Fig. 2.2 B). Examination of sections of uterine tissue obtained from the null animals at later time points, such as 22 and 24 h, also did not indicate any substantial immunostaining for P-His3 (data not shown), confirming that mitosis is not delayed but truly impaired in the mutant stromal cells. The reduced mitotic activity of the C/EBPβ-null uterine stromal cells indicated that these cells, which are able to undergo DNA replication, are arrested in the cell cycle prior to mitosis, presumably at the G2-M boundary.

C/EBPβ controls the expression of cyclin B at G2-M transition of the cell cycle

The cell cycle is primarily regulated by a complex interplay of cyclins, cyclin-dependent kinases (Cdks) and cyclin-dependent kinase inhibitors (28-31). We, therefore, investigated whether C/EBPβ controls the proliferation of stromal cells by regulating the

expression of certain of the molecules that specifically control various cell cycle checkpoints. Uteri were collected from WT and C/EBPβ-null mice at different times after experimentally induced decidualization and the stromal cells were isolated from these tissue samples. We then monitored the expression of specific cyclins and Cdks that are hallmarks of G1-S and G2-M phases.

Previous studies showed that cyclin D3 and cyclin E1 are predominant D-type and E-type cyclins, respectively, in mouse stromal cells (32). As shown in Fig. 3, panels A-D, the expression levels of mRNAs corresponding to cyclin D3, cyclin E1, Cdk4 and Cdk2, which are critical regulators of G1-S transition, were elevated in uterine stromal cells of both WT and C/EBPβ-null mice at 15 h following decidual stimulation. We also noted that their expression declined at 18 h in WT as well as C/EBPβ-null mice as the cells completed the S phase and entered the G2 phase. There was no statistically significant difference between the expression levels of these cyclins and cdks in uterine stromal cells of WT and C/EBPβ-null mice. Additionally, no significant alteration in the expression level of the mRNA encoding cdc25A, a phosphatase that regulates Cdk4 activity, was observed between WT and C/EBPβ-null uterine stromal cell (Fig. 2.3, panel E). Collectively, these results are consistent with our observation in Fig. 2.1 that the lack of C/EBPβ does not affect the advancement of the stromal cells through the S phase of the cell cycle.

We next examined the expression of several factors such as Cyclin B, Cyclin A, and Cdk1, which are critical for the entry of the stromal cells into the mitotic phase. During the G2-M transition, binding of cyclin A or cyclin B to Cdk1 induces phosphorylation and activation of this kinase, which plays a critical role in this process. As shown in Fig. 2.4, panels A and B, no significant alteration in stromal expression of cyclin A or Cdk1 mRNA was seen in the C/EBPβ-null uteri relative to the WT uteri at either 16 or 20 h following decidual stimulation. In contrast, the expression of cyclin B1 and cyclin B2 mRNAs was significantly reduced in C/EBPβ-null uterine stromal cells, particularly at 20 h following decidual stimulation, when the cells are supposed to enter the mitotic phase (Fig. 2.4, panels C and D). It is pertinent to mention here that cyclin B2 mRNA is expressed at a higher level compared to cyclin B1 mRNA in the uterine stromal

cells (Wei Wang, unpublished data). Whereas the mRNA level of cyclin B1 decreased by ~50% in uterine stromal cells lacking C/EBPβ, that of cyclin B2 decreased by more than 80%.

To further validate the regulation of cyclin B2 by C/EBPβ, we analyzed its expression at the protein level. As shown in the western blot in Fig. 2.4E, the signal(s) of cyclin B2 was drastically reduced in cell lysates prepared from stromal cells obtained from C/EBPβ-null uteri subjected to experimentally induced decidualization. We also monitored cyclin B2 expression using immunocytochemistry in primary uterine stromal cells undergoing decidualization *in vitro*. Under these conditions, we observed a strong nuclear expression of cyclin B2 in WT stromal cells undergoing mitosis at 20 h following the initiation of the decidualization process (Fig. 2.4F). This immunostaining of cyclin B2 was markedly diminished in the stromal cells isolated from C/EBPβ-null uteri and cultured under similar conditions. Our results supported the concept that C/EBPβ is a critical regulator of the B-type cyclins, B1 and B2, which control the transition of the uterine stromal cells into mitosis.

C/EBPβ manifests its transcriptional function by binding to the CCAAT motifs at the promoter regions of its target genes to directly influence their rate of transcription. *In silico* analysis of the promoter of cyclin B2, using the bioinformatics softwares TESS, TFSearch and Consite, revealed that it contains four candidate C/EBPβ binding sites within the 2 kilo-base 5‘-flanking region of the gene at approximately -403, -686, -930 and –1651 relative to the transcription start site. To test whether C/EBPβ actually binds to one or more of these sites, we employed chromatin immunoprecipitation using an antibody specific for C/EBPβ. As shown in Fig. 2.5A, when primary cultures of uterine stoma cells were subjected to in vitro decidualization, no significant binding of C/EBPβ was detected at the –403, –686 and –1651 sites of the cyclin B2 promoter. In contrast, a relatively strong binding of this transcription factor was observed at the cyclin B2 promoter region -930.

To further ascertain that the –930 region of the cyclin B2 promoter represents a functional C/EBPβ binding site, we employed a dominant-negative mutant of C/EBPβ.

Previous studies showed that this mutant, A-C/EBP, which lacks the DNA binding domain but retains the dimerization domain, inhibits DNA binding and transcriptional function of endogenous C/EBPβ (33). Primary stromal cells were transduced with recombinant adenovirus expressing A-C/EBP (Fig. 2.5B) or control adenovirus expressing GFP. As shown in Fig. 2.5C, A-C/EBP, which inhibits DNA binding and transcriptional activity of endogenous C/EBPβ, abolishes the occupancy of the –930 region of the cyclin B2 promoter by this transcription factor. These data demonstrated that the -930 region of the cyclin B2 promoter represents a bonafide C/EBPβ binding site and it likely mediates the direct regulation of the expression of this cyclin by C/EBPβ.

Loss of cdc25C expression in C/EBPβ-null uterine stromal cells may contribute to decreased Cdk1 activity leading to G2-M arrest

cdc25C is a cell cycle regulatory phosphatase, which is a critical regulator of Cdk1 activity. It maintains Cdk1 activity during mitosis via the reversal of inhibitory phosphorylations at Thr14 and Tyr15 of Cdk1. We observed that the level of cdc25C mRNA was markedly down regulated in C/EBPβ-null uterine stromal cells relative to the WT stromal cells at the time of G2-M phase transition (Fig. 2.6A). This result was further confirmed upon analysis of the levels of cdc25C protein in uterine stromal cells subjected to *in vitro* decidualization. Immunocytochemical analysis revealed that the expression of cdc25C was significantly reduced in the C/EBPβ-null uterine stromal cells, while Cdk1 was expressed at similar levels in WT and mutant stromal cells (Fig. 2.6, panels B and C),

To ascertain that the down regulation of cdc25C expression in C/EBPβ-null uterine stromal cells indeed affects the inhibitory phosphorylation status of Cdk1 at Thr14/Tyr15, we performed immunocytochemistry using an antibody which specifically recognizes this modification. Our results confirmed that the level of p-Cdk1 was higher in C/EBPβ-null uterine stromal cells relative to the WT cells (Fig. 2.6, panel D). These results are consistent with the hypothesis that the regulation of the expression of cdc25C represents an additional mechanism by which C/EBPβ controls the activity of Cdk1 during mitotic entry.

Elevated expression of cell cycle inhibitors in C/EBβ-null uterine stromal cells

It is well documented that cyclin-dependant kinase inhibitors p21 and p27, and the cell cycle suppressor p53 inhibit the activity of various cyclin-Cdk complexes, resulting in the arrest of the cell cycle at the G1-S and G2-M checkpoints (34-39). To determine whether C/EBPβ controls uterine stromal proliferation by regulating the expression of these cell cycle suppressors, we analyzed the levels of their mRNAs in WT and C/EBPβ-null uterine stromal cells by real-time PCR (Fig. 2.7A). We found that the expression of p53 mRNA was modestly increased in C/EBPβ-null stromal cells at 16 h following decidual stimulation, while the levels of mRNAs corresponding to p21 and p27 did not change significantly at this time point (Fig. 7, panels A-C). In contrast, the levels of p21, p27 and p53 mRNAs were markedly elevated in the mutant stromal cells at 20 h following the decidual stimulation when G2-M phase transition is supposed to occur. Similar changes in their protein levels were confirmed by Western blot analysis (Fig. 2.7, panel D). These results indicated that C/EBPβ-mediated stromal cell proliferation during decidualization is associated with the suppression of expression of p21, p27 and p53 proteins during the G2-M phase transition. Loss of C/EBPβ expression in C/EBPβ-null stromal cells results in the up regulation of these inhibitors, which then repress the activity of cyclin B-Cdk1 complex and contribute to the blockade of stromal cell mitosis (Fig. 2.8).

2.5 Discussion

Previous studies indicated that C/EBPβ is a critical regulator of cell proliferation and/or differentiation in multiple tissues including the liver, adipose tissue, immune system, skin and mammary gland (18;40-47). In the liver and breast, it promotes growth and proliferation of hepatocytes and mammary epithelium, respectively (45-47). In contrast, it exerts an antiproliferative action in epidermal keratinocytes and contributes to their growth arrest (42). C/EBPβ, therefore, is implicated in both positive and negative control of the cell cycle, depending on the cellular context.

This paper addresses the role of C/EBPβ in uterine stromal proliferation. During the decidualization phase of pregnancy, steroid hormone-primed uterine stromal cells undergo proliferation and mitotic expansion during the first 24-48 h following decidual stimulation (19-22;48). The precise mechanisms by which E and P regulate stromal proliferation and differentiation in the uterus remain unclear. We have previously reported that C/EBPβ is a mediator of the biological actions of E and P in mouse uterus (13). Its expression is robustly induced in the decidual tissue during normal as well as experimentally induced decidualization (13). The expression of C/EBPβ in decidual cells arises from a complex interplay of E and P within the uterine compartments. Although a transient rise in the level of E in the pre-implantation period induces C/EBPβ in the epithelial and stromal cells of pregnant uterus, PR becomes a critical regulator of this gene as the stromal cells become progressively differentiated (13). In this paper, we provide evidence that C/EBPβ plays a central role in controlling the stromal cell cycle during decidualization. This study, therefore, provides a molecular explanation of the gene regulatory pathways via which the steroid hormones execute the growth and expansion of uterine stromal population at the onset of decidualization.

It is well established that the proliferation of animal cells proceeds through various stages of the cell cycle: G1, S, G2, and M, and is controlled at distinct checkpoints (28;31;49;50). Progression through each phase of the cell cycle is under the strict control of multiple regulatory factors, such as, cyclins, cyclin-dependent kinases (Cdks), and Cdk-inhibitors (28;31;49;50). Cyclin levels fluctuate dramatically through the cell cycle as a consequence of changes in transcription and ubiquitin-mediated degradation. Different cyclins bind specifically to different Cdks, which are serine/threonine protein kinases, to form distinct regulatory complexes at specific phases of the cell cycle and thereby drive the cell from one phase to another. The association of D-type cyclins with Cdk4 or Cdk6 is important for G1-S transition. Binding of B-type cyclins to Cdk1 allows phosphorylation of this enzyme by the Cdk-activating kinase (CAK) and its activation, which plays an essential role in G2-M transition. Our present study showed that the C/EBPβ-null stromal cells are able to enter the cell cycle and proceed through the S phase (DNA synthesis) in response to a decidual stimulation.

Consistent with this finding, we did not observe any significant change in the expression of cyclin D, cyclin E, Cdk2, Cdk4 and cdc25A, which control G1 to S phase transition and S phase activity, in WT versus mutant stromal cells.

Strikingly, the C/EBPβ-null uterine stromal cells exhibited impaired mitosis. Our results suggested that the stromal cells are arrested at the G2-M checkpoint of the cell cycle in the absence of C/EBPβ. We considered the possibility that the expression of one or more critical regulators of G2-M transition is lost in the C/EBPβ-null uterine stromal cells. We found that the mRNAs corresponding to the B-type cyclins B1 and B2 are markedly down regulated in the mutant stromal cells compared to the WT cells. Our studies confirmed that the level of cyclin B2 protein declined sharply in the C/EBPβ-null stromal cells. Previous reports indicated that multiple CCAAT box-like motifs are located at human or mouse cyclin B2 promoter (51;52). To explore whether the C/EBPβ directly binds to the cyclin B2 promoter and regulates its expression, we employed the ChIP procedure. Our results demonstrated that C/EBPβ binds strongly to a specific region in the cyclin B2 promoter that contains a CCAAT-like motif. This finding is consistent with the hypothesis that C/EBPβ is a direct regulator of transcription of the cyclin B2 gene.

Previous studies showed that targeted deletion of cyclin B1 gene resulted in embryonic lethality in mice (53). In contrast, mutant mice lacking cyclin B2 are viable and both sexes are fertile. However, mating of homozygous mice cyclin B2-null mice produced markedly reduced litter size, although the reason for this sub-fertility remain unclear (53). It was thought that cyclin B1, whose expression overlaps with that of cyclin B2 in many tissues, compensates for the loss of cyclin B2 in the mutant mice. We found that both cyclins B1 and B2 are expressed in the uterine stromal cells during pregnancy, although the cyclin B2 levels are significantly higher than that of cyclin B1. It is important to note that C/EBPβ controls the expression of both B-type cyclins. Therefore, the absence of this transcription factor would lead to a drastic reduction in the overall levels of B-type cyclins in the uterine stromal cells, consistent with the observed block in their mitotic activity.

A well-established mechanism to control entry into mitosis is to regulate the activity of the cyclin B/Cdk1 complex (31). The function of Cdk1 is tightly controlled by modulation of phosphorylation and dephosphorylation of this kinase (50). Binding of cyclin B triggers phosphorylation of Cdk1 by CAK, which leads to its activation. On the other hand, the Cdk1 activity is greatly reduced by inhibitory phosphorylation at Thr14 and Tyr15 by the Wee1 and Myt1 kinases. Cdc25C, a cell cycle regulatory phosphatase, maintains the active state of Cdk1 through dephosphorylation of the Thr14 and Tyr15 sites. We investigated whether C/EBPβ regulates the Cdk1 activity during mitosis by altering the expression of any of the kinases and phosphatases that control Cdk1 activity. While no change was seen in the expression of the kinases that modulate Cdk1 function (data not shown), we found that the expression of cdc25C is greatly reduced in the C/EBPβ-null uterine stromal cells. As expected, concomitant with the decrease in the level of cdc25C, there was a significant up regulation in the level of phospho-Cdk1 (Thr 14/Tyr 15), which likely contributes to the mitosis block.

Interestingly, we noted that the levels of several well-known cell cycle suppressors, p21, p27 and p53, were markedly elevated specifically at the time of G2-M transition. This finding uncovered additional mechanisms by which C/EBPβ controls stromal proliferation during decidualization progression. p53, an important cell cycle repressor, is a transcription factor induced and activated in response to various stress signals or DNA damage events (54). Numerous studies have shown that p53 is able to trigger cell cycle arrest at G1-S and G2-M checkpoints via different mechanisms involving its downstream transcriptional targets such as p21. Our results are in agreement with earlier findings of Yoon *et al* who demonstrated a striking increase of p53 expression and function in the keratinocytes of C/EBPβ-null mice, indicating that C/EBPβ has a role in the negative regulation of p53 in normal keratinocytes (55). Previous reports also indicated that C/EBPβ is able to bind efficiently to the p53 promoter and suppress its transcription (56). Conversely, p53 can repress the transcriptional activity of C/EBPβ by physically interacting with its C-terminal domain (57). This two-way cross-talk between C/EBPβ and p53 is likely to influence the

expression of both p53 and C/EBPβ and their downstream genes during the stromal decidualization process.

p21, the transcriptional target of p53, binds directly to a broad spectrum of cyclin-Cdk complexes and inhibits their function (37). Another well-known inhibitor, p27, also functions by binding to the G1/S and G2/M cyclins and interfering with their activities (38;39). Interestingly, the levels of p27 and p53-induced p21 did not change significantly in C/EBPβ-null uterine stromal cells at the time of G1-S transition following a decidual stimulation. We observed that the levels of p27 and p21 are elevated in the mutant stromal cells specifically at the time of G2/M transition, indicating that these cell cycle inhibitory molecules contribute to the G2/M block of stromal proliferation seen in the C/EBPβ-null mice.

A comparison of the mechanisms by which C/EBPβ controls uterine stromal proliferation with previously reported mechanisms of its action in other tissues, such as the liver, adipose tissue and mammary gland, revealed interesting differences. In C/EBPβ-null female mice, mammary epithelial cells fail to proliferate in response to steroid hormones, leading to an impaired ductal morphogenesis (46;47). It was shown that loss of C/EBPβ in this tissue markedly reduced the expression of cyclin E, which contributed to a G1/S arrest (58). Additionally, the mutant mammary epithelial cells showed decreased cdc25A expression and increased p27 stability. Loss of C/EBPβ expression in models of liver regeneration showed a similar defect in cell cycle progression through the S phase, and it was mainly attributed to a lack of expression of cyclin E (45). In the C/EBPβ-null stromal cells, however, we did not find any defect in the G1-S transition as the cells were able to proceed through the S phase before their arrest at the G2-M checkpoint. These findings suggested that C/EBPβ regulation of cell proliferation is cell context specific and a unique regulatory strategy involving the control of the mitosis step operates during uterine stromal proliferation during decidualization.

In summary, this study provides new insights into the mechanisms by which C/EBPβ mediates the effects of steroid hormones during decidualization. Our results show that C/EBPβ is a key regulator of the stromal proliferation and it operates by

controlling the expression and function of multiple cell cycle regulatory molecules that control the G2-M transition of the decidualizing stromal cells. This study is important because it provides a mechanistic understanding of the hormone-regulated pathways that control decidualization, which is a prerequisite for the successful establishment of pregnancy.

2.6 Acknowledgements

The authors are grateful to Dr. Peter Johnson of NCI/NIH who kindly provided the C/EBPβ-null mice. The recombinant adenovirus containing A-C/EBP was kindly provided by Dr. Charles Vinson of NCI/NIH. This work was supported by the Eunice Kennedy Shriver NICHD/NIH through cooperative agreement U54 HD055787 as part of the Specialized Cooperative Centers Program in Reproduction and Infertility Research.

2.7 Figures and legends

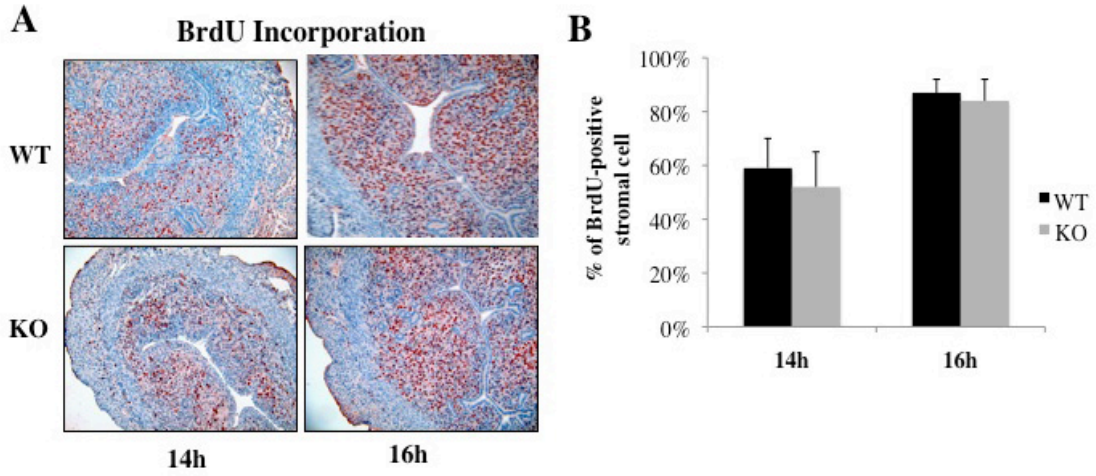

Figure 2.1 Examination of S phase activity of uterine stromal cells of C/EBPβ-null mice using BrdU immunostaining

Ovariectomized WT and C/EBPβ-null mice were subjected to artificial decidual stimulation and uteri were collected at different time points following application of the stimulus. A: Mice were given BrdU injection at 12 and 14 h after stimulation. After a 2 h pulse, the uterine tissues were collected at 14 and 16 h. The tissues were fixed, embedded in paraffin, sectioned and subjected to IHC using an antibody specific for BrdU. At least three WT or C/EBPβ-null mice were used at each time point and representative data are shown.

B. The number of cells in sections of C/EBPβ-null uteri that are immunoreactive to BrdU-antibody was quantitated and compared with those in WT uteri. Data were expressed as average +/- S.D of three separate measurements.

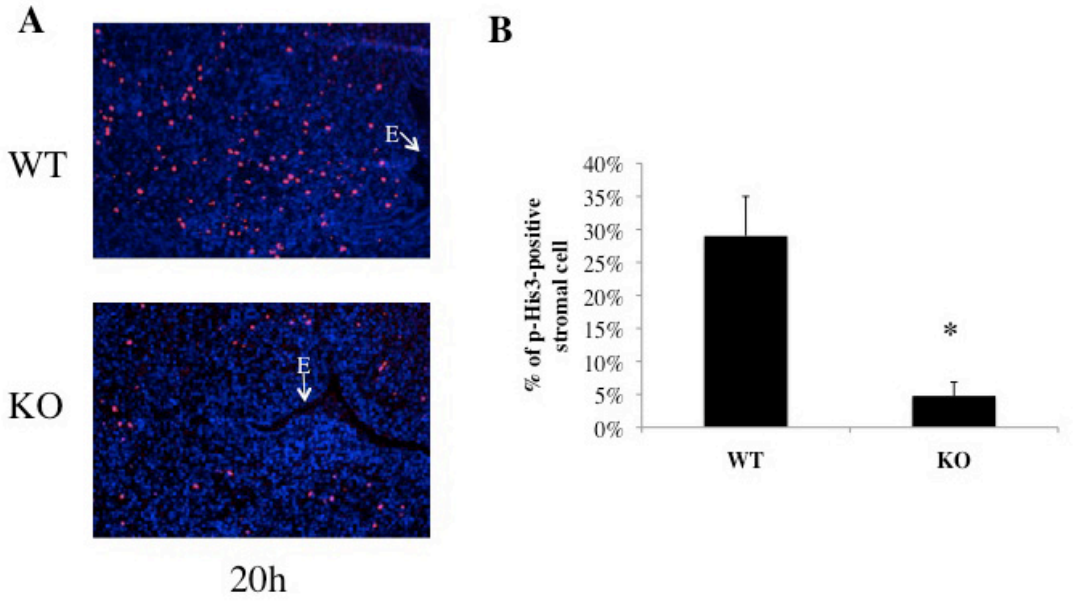

Figure 2.2 Stromal proliferation is blocked at G2-M transition in C/EBPβ-null uteri

Ovariectomized WT and C/EBPβ-null mice were subjected to artificial decidual stimulation and uteri were collected at different time points after application of the stimulus. A. Sections of uterine tissues obtained at 20 h after artificial decidual stimulation were subjected to immunofluorescence using an antibody specific for phosphorylated Ser 10 of histone 3. E and S denote surface epithelium and stroma, respectively. B. The number of phospho-histone 3-positive cells in C/EBPβ-null uteri was quantitated and compared with those in WT uteri. Data were expressed as average +/- S.D of three separate measurements.

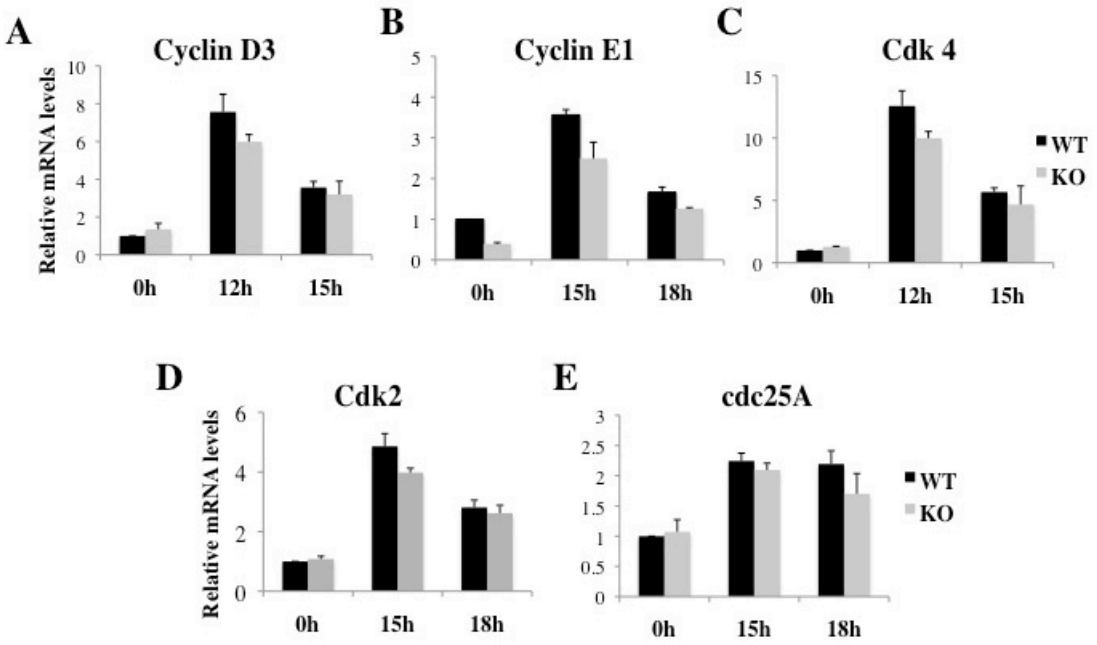

Figure 2.3. Analysis of expression of S phase-specific cell cycle regulators in uterine stromal cells of C/EBPβ-null mice

Ovariectomized WT and C/EBPβ-null mice were subjected to artificial decidual stimulation. Stromal cell were isolated at different times after application of the stimulus and total RNA was prepared from these cells. Five WT or C/EBPβ-null mice were used at each time point and the uteri of each category were pooled. The expression levels of mRNAs corresponding to cyclin D3, cyclin E1, Cdk2, Cdk4 and cdc25A were monitored by real time PCR, using gene-specific primers. All PCR data were normalized with respect to the mRNA level of 36B4. The relative fold inductions of the expression of these genes at different times were determined after setting that of 0 h WT cells at 1.0. The values represent average +/- S.D of three separate measurements. WT: black bar and C/EBPβ-null: grey bar.

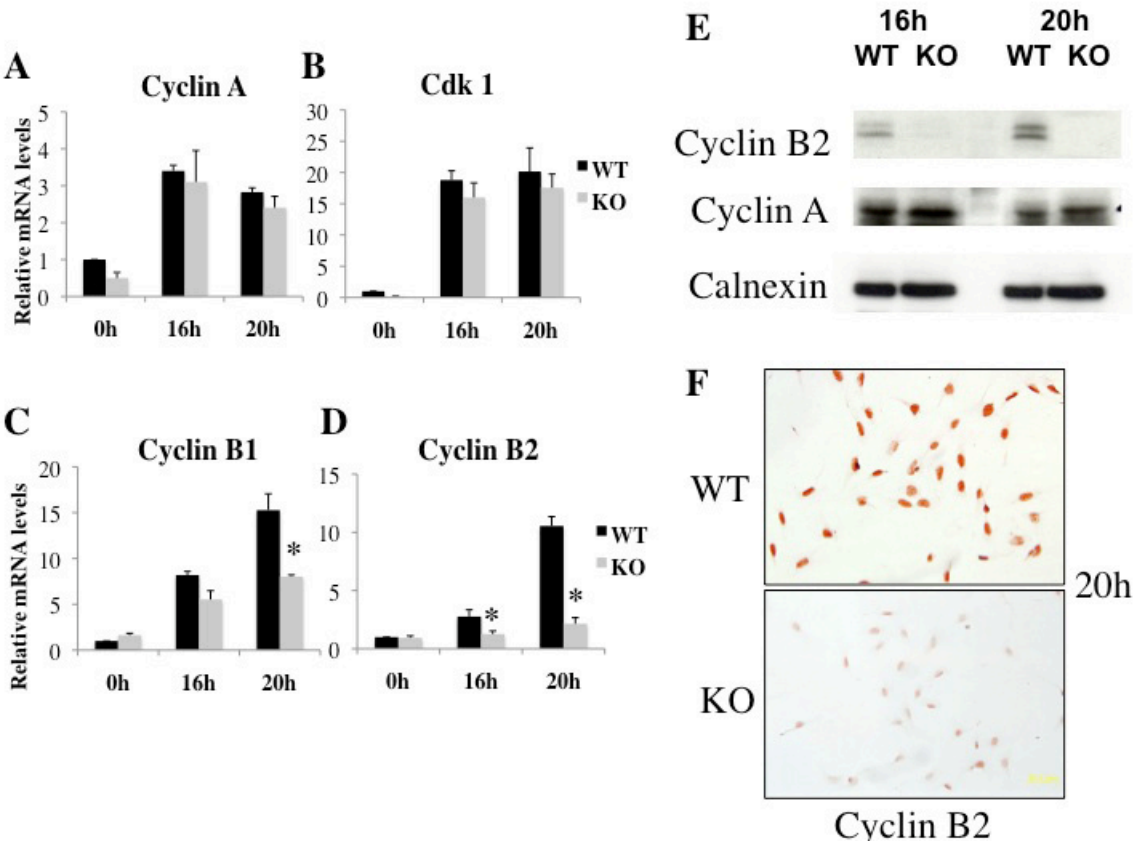

Figure 2.4 C/EBPβ controls the expression of the cell cycle regulators at G2-M phase. Uterine stromal cells were isolated from WT and C/EBPβ-null mice at different times after application of artificial decidualization stimulus.

A-D. Five WT or C/EBPβ-null mice were used at each time point and the uteri of each category were pooled. The expression levels of mRNAs corresponding to cyclin A, Cdk1, cyclin B1 and cyclin B2 were monitored by real time PCR, using gene-specific primers. All PCR data were normalized with respect to the mRNA level of 36B4. The relative fold inductions of the expression of these genes at different times were determined after setting that of 0 h WT cells at 1.0. The values represent average +/- S.D of three separate measurements. WT: black bar and C/EBPβ-null: grey bar. * indicates statistically significant change ($P<0.05$).

E. Stromal cells were isolated from WT or C/EBPβ-null uteri collected at 16 and 20 h after artificial decidual stimulation. The cells were lysed, analyzed by SDS-PAGE and

Fig. 2.4 (continued) C/EBPβ controls the expression of the cell cycle regulators at G2-M phase. Uterine stromal cells were isolated from WT and C/EBPβ-null mice at different times after application of artificial decidualization stimulus

subjected to Western blotting using antibodies directed against cyclin B2 and cyclin A. Immunostaining of calnexin served as a loading control.

F. Stromal cells were isolated from WT or C/EBPβ-null uteri collected immediately after artificial decidual stimulation, and allowed to attach to slides for 2 h. The cells were cultured *in vitro* in medium supplemented with P (1 μM) and E (10 nM). At 20 h, the cells were fixed and subjected to immunocytochemical staining using a cyclin B2 antibody.

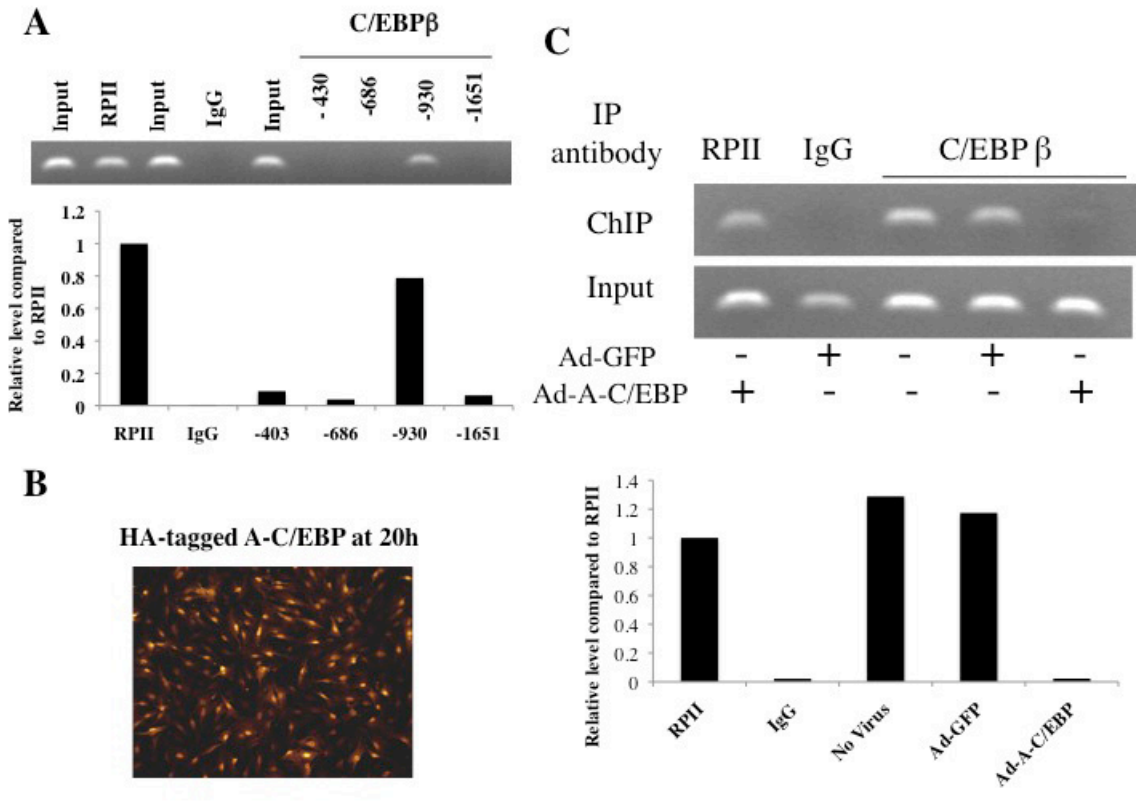

Figure 2.5 Regulation of cyclin B2 expression by C/EBPβ.

A. Stromal cells were isolated from WT uteri collected immediately after artificial decidual stimulation, and allowed to attach to slides for 2 h. The cells were cultured *in vitro* in medium supplemented with P (1 μM) and E (10 nM). At 20 h, the cells were treated with formaldehyde, DNA-protein complexes were cross-linked and subjected to chromatin immunoprecipitation (ChIP) assay as described in the Materials and methods. Chromatin immunoprecipitates obtained using antibodies against RNA polymerase II and mouse IgG served as positive and negative controls, respectively. DNAs isolated from the immunoprecipitates were used as templates for regular PCR (upper panel) and real time PCR (lower panel) using various primer sets to amplify specific regions of the cyclin B2 promoter. The nucleotide positions of the candidate C/EBP binding sites within the four promoter regions analyzed by ChIP are indicated. The experiment was repeated twice and representative data are shown.

(Fig. 2.5 continued)B. Stromal cells were isolated from WT uteri collected immediately after artificial decidual stimulation, and allowed to attach to slides for 2 h. Then the cells were transduced with adenovirus expressing dominant-negative A-C/EBP (MOI is 10:1). 20 h after virus addition, the cells were examined by immunofluorescence using an anti-HA antibody.

C. Stromal cells were isolated from WT uteri collected immediately after artificial decidual stimulation, and allowed to attach to slides for 2 h. Then the cells were cultured in medium supplemented with P (1 μM) and E (10 nM) and transduced with adenovirus expressing dominant-negative A-C/EBP or control virus (Ad-GFP) (MOI is 10:1) or no virus treated. After 20 h transduction, Cells were treated with formaldehyde and ChIP assays were performed as described in the Materials and methods. Chromatin immunoprecipitates obtained using antibodies against RNA polymerase II and mouse IgG served as positive and negative controls, respectively. DNAs isolated from the immunoprecipitates were used as templates for regular PCR (upper panel) and real time PCR (lower panel) to determine promoter occupancy by C/EBPβ at the -930 site of the Cyclin B2 promoter. The experiment was repeated twice and representative data are shown.

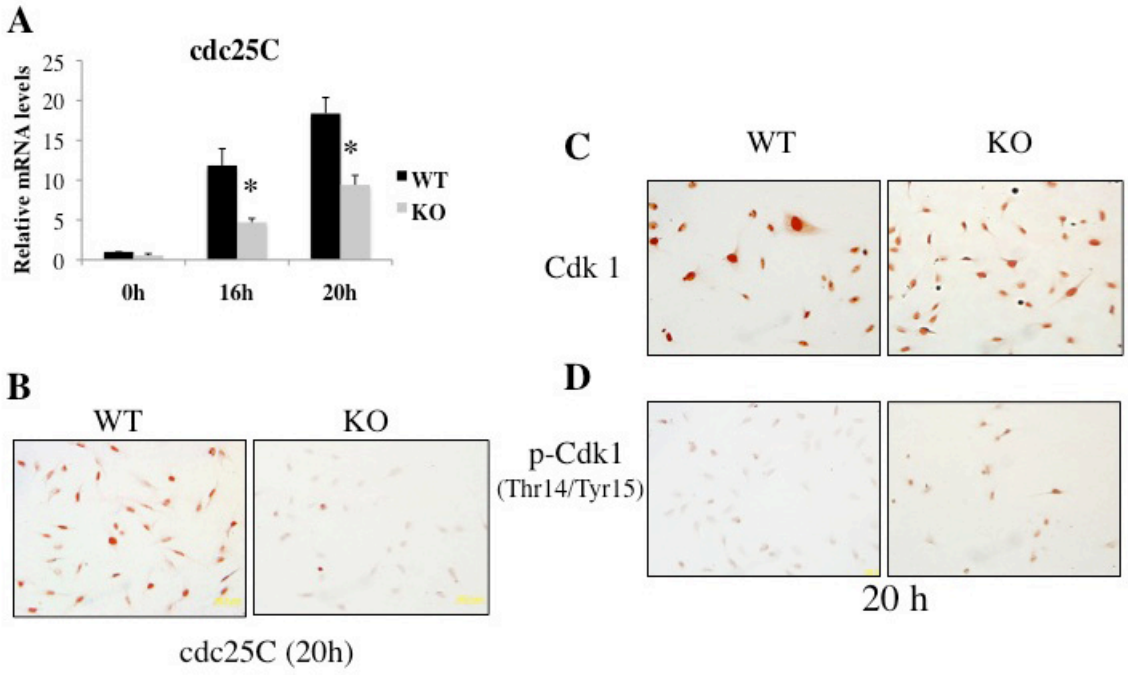

Figure 2.6 C/EBPβ regulates the activity of Cdk1.

A. Ovariectomized WT and C/EBPβ-null mice were subjected to artificial decidual stimulation. Stromal cell were isolated at different times after application of the stimulus and total RNA was prepared from these cells. Five WT or C/EBPβ-null mice were used at each time point and the uteri of each category were pooled. The expression levels of Cdk1 mRNAs were monitored by real-time PCR, using gene-specific primers. * indicates statistically significant change ($P<0.05$).

B-D. Stromal cells were isolated from WT or C/EBPβ-null uteri collected immediately after artificial decidual stimulation, and allowed to attach to slides for 2 h. The cells were cultured *in vitro* in medium supplemented with P (1 μM) and E (10 nM). At 20 h, the cells were fixed and subjected to immunocytochemical staining using antibodies against Cdk1, cdc25C and phospho-Cdk1 (Thr14/Tyr15).

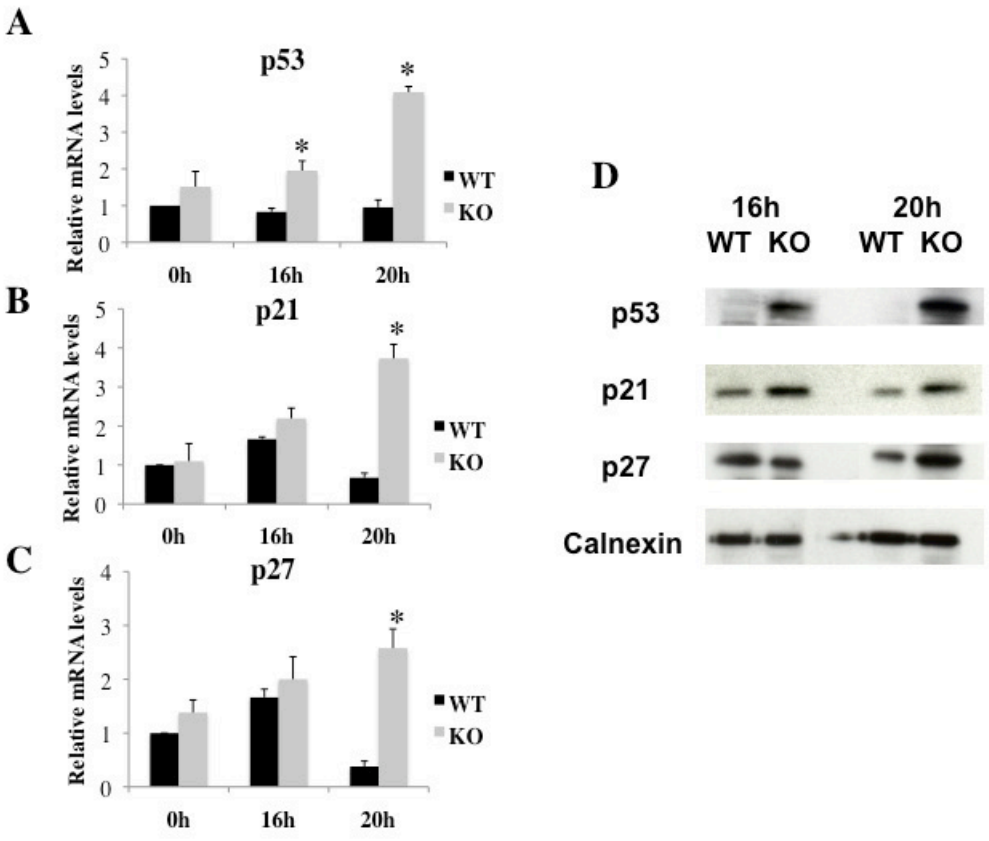

Figure 2.7 Increased expressions of p53, p21 and p27 in C/EBPβ-null uteri

A-C. Ovariectomized WT and C/EBPβ-null mice were subjected to artificial decidual stimulation. Stromal cell were isolated at different times after application of the stimulus and total RNA was prepared from these cells. Five WT or C/EBPβ-null mice were used at each time point and the uteri of each category were pooled. The expression levels of mRNAs corresponding to p53, p21, and p27 were monitored by real time PCR, using gene-specific primers. All PCR data were normalized with respect to the mRNA level of 36B4. The relative fold inductions of the expression of these genes at different times were determined after setting that of 0 h WT cells at 1.0. The values represent average +/- S.D of three separate measurements. WT: black bar and C/EBPβ-null: grey bar. * indicates statistically significant change ($P<0.05$).

D, Stromal cells were isolated from WT or C/EBPβ-null uteri collected at 16 and 20 h after artificial decidual stimulation. The cells were lysed, analyzed by SDS-PAGE and

Fig. 2.7 (continued) Increased expressions of p53, p21 and p27 in C/EBPβ-null uteri subjected to Western blotting using antibodies directed against p53, p21, p27. Calnexin immunostaining served as a loading control.

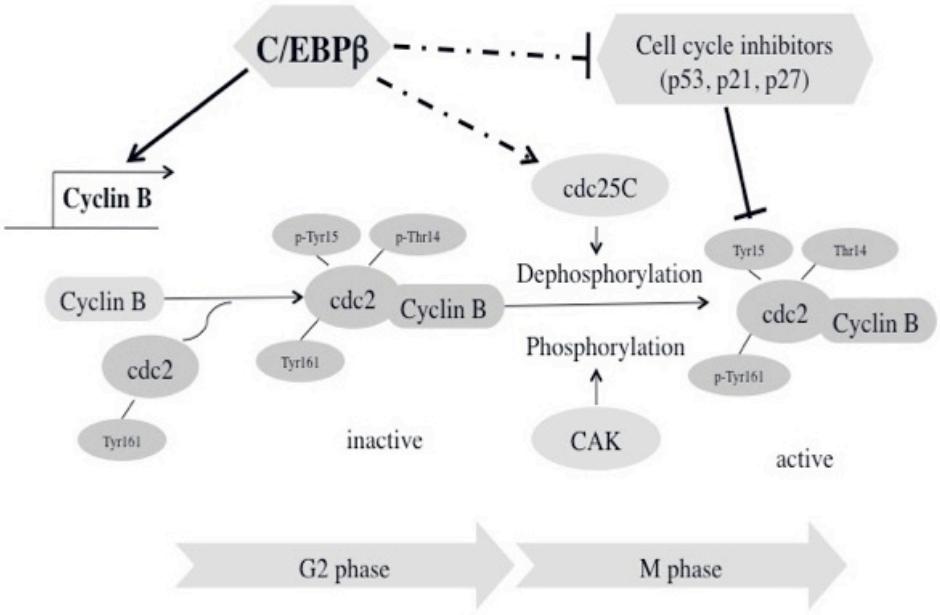

Figure 2.8 A schematic of the mechanisms underlying C/EBPβ regulation of endometrial stromal proliferation during decidualization

C/EBPβ plays a central role in stromal proliferation during decidualization by controlling the expression of multiple cell cycle regulatory molecules, such as cyclin B, phosphatase cdc25C, and the inhibitory factors p53, p21 and p27, during G2-M transition of the cell cycle. CAK is a kinase that induces activating phosphorylation in Cdk1. Wee1 and Myt1 are kinases that induce inhibitory phosphorylations in Cdk1.

References

1. **Dey SK, Lim H, Das SK, Reese J, Paria BC, Daikoku T, Wang H** 2004 Molecular cues to implantation. Endocr Rev 25:341-373
2. **Lee KY, Jeong JW, Tsai SY, Lydon JP, DeMayo FJ** 2007 Mouse models of implantation. Trends Endocrinol Metab 18:234-239
3. **Yoshinaga K** 1988 Uterine receptivity for blastocyst implantation. Ann NY Acad Sci, 1988, pp 424-431
4. **Parr MB, Parr EL** 1989 The Implantation Reaction. New York: Plenum Press
5. **Phychoyos A** 1973 Endocrine control of egg implantation. Washington DC: American Physiological Society
6. **Weitlauf HM** 1994 Biology of implantation. New York
7. **Finn CA** 1977 The implantation reaction. In: Wynn RM ed. Biology of the Uterus. New York: Plenum Press; 246-308
8. **DeFeo VJ** 1967 Decidualization. In: Wynn RM ed. Cellular biology of the uterus. Amsterdam: North Holland; 191-290
9. **Glasser SR, Mulholland J, Mani SK, Julian J, Munir I, Lampelo S, Soares MJ** 1991 Blastocyst-endometrial relationships: reciprocal interactions between uterine epithelial and stromal cells and blastocysts. Trophoblast Research 5:229-280
10. **Gu Y, Gibori G** 1999 Deciduoma: Academic Press
11. **Irwin JC, Giudice D** 1999 Encyclopedia of Reproduction. New York: Acadmic Press
12. **Cross JC, Werb Z, Fisher SJ** 1994 Implantation and the placenta: key pieces of the developmental puzzle. Science 266:1508-1518
13. **Mantena SR, Kannan A, Cheon YP, Li Q, Johnson PF, Bagchi IC, Bagchi MK** 2006 C/EBPbeta is a critical mediator of steroid hormone-regulated cell proliferation and differentiation in the uterine epithelium and stroma. Proceedings of the National Academy of Sciences of the United States of America 103:1870-1875
14. **Bagchi MK, Mantena SR, Kannan A, Bagchi IC** 2006 Control of uterine cell proliferation and differentiation by C/EBPbeta: functional implications for establishment of early pregnancy. Cell Cycle 5:922-925
15. **Wedel A, LomsZiegler-heitbrock HW** 1995 The C/EBP family of transcription factors. Immuno Biol 9:171-185
16. **McKnight SL** 2001 McBindall--a better name for CCAAT/enhancer binding proteins? Cell 107:259-261

17. **Ramji DP, Foka P** 2002 CCAAT/enhancer-binding proteins: structure, function and regulation. Biochem J 365:561-575

18. **Sterneck E, Tessarollo L, Johnson PF** 1997 An essential role for C/EBPbeta in female reproduction. Genes & development 11:2153-2162

19. **Das RM, Martin L** 1978 Uterine DNA synthesis and cell proliferation during early decidualization induced by oil in mice. J Reprod Fertil 53:125-128

20. **Moulton BC** 1979 Effect of progreterone on DNA, RNA, and protein synthesis during preimplantation procursors of stromal cell differentiation during decidualization. Endocrinology 115:1302-1307

21. **Moulton BC, Keonig BB** 1984 Uterine deoxyribonucleic acid synthesis during preimplantation procursors of stromal cell differentiation during decidualization. Endocrinology 115:1302-1307

22. **Dey SK** 1996 Implantation. New York: Lipincott-Raven

23. **Cheon YP, DeMayo FJ, Bagchi MK, Bagchi IC** 2004 Induction of cytotoxic T-lymphocyte antigen-2beta, a cysteine protease inhibitor in decidua: a potential regulator of embryo implantation. The Journal of biological chemistry 279:10357-10363

24. **Li Q, Kannan A, Wang W, Demayo FJ, Taylor RN, Bagchi MK, Bagchi IC** 2007 Bone morphogenetic protein 2 functions via a conserved signaling pathway involving Wnt4 to regulate uterine decidualization in the mouse and the human. The Journal of biological chemistry 282:31725-31732

25. **Li Q, Cheon YP, Kannan A, Shanker S, Bagchi IC, Bagchi MK** 2004 A novel pathway involving progesterone receptor, 12/15-lipoxygenase-derived eicosanoids, and peroxisome proliferator-activated receptor gamma regulates implantation in mice. The Journal of biological chemistry 279:11570-11581

26. **Zhao X, Zhuang S, Chen Y, Boss GR, Pilz RB** 2005 Cyclic GMP-dependent protein kinase regulates CCAAT enhancer-binding protein beta functions through inhibition of glycogen synthase kinase-3. The Journal of biological chemistry 280:32683-32692

27. **Hendzel M, Wei Y, Mancini MA, Hooser AV, Ranalli T, Brinkley BR, Bazett-Jones, Allis CD** 1997 Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. Chromosoma 106:348-360

28. **Alberts B** 2002 Molecular Biology of the Cell. New York: Science

29. **Harper JV** 2005 Synchronization of cell populations in G1/S and G2/M phases of the cell cycle. Methods Mol Biol 296:157-166

30. **G B** 2004 Cyclins, cyclin-dependent kinases and cylcin dependent kinase inhibitors. . Methods in Molecular Biology 296:291-298

31. **Nurse P** 2000 A long twentieth century of the cell cycle and beyond. Cell 100:71-78

32. **Das SK, Lim H, Paria BC, Dey SK** 1999 Cyclin D3 in the mouse uterus is associated with the decidualization process during early pregnancy. J Mol Endocrinol 22:91-101

33. **Zhang JW, Tang QQ, Vinson C, Lane MD** 2004 Dominant-negative C/EBP disrupts mitotic clonal expansion and differentiation of 3T3-L1 preadipocytes. Proceedings of the National Academy of Sciences of the United States of America 101:43-47

34. **Naidu KA, Fang Q, Cheng JQ, Nicosia SV, Coppola D** 2007 P53 enhances ascorbyl stearate-induced G2/M arrest of human ovarian cancer cells. Anticancer Res 27:3927-3934

35. **Kaushal N, Bansal MP** 2007 Inhibition of CDC2/Cyclin B1 in response to selenium-induced oxidative stress during spermatogenesis: potential role of Cdc25c and p21. Mol Cell Biochem 298:139-150

36. **Taylor BF, McNeely SC, Miller HL, Lehmann GM, McCabe MJ, Jr., States JC** 2006 p53 suppression of arsenite-induced mitotic catastrophe is mediated by p21CIP1/WAF1. J Pharmacol Exp Ther 318:142-151

37. **Smits VA, Klompmaker R, Vallenius T, Rijksen G, Makela TP, Medema RH** 2000 p21 inhibits Thr161 phosphorylation of Cdc2 to enforce the G2 DNA damage checkpoint. The Journal of biological chemistry 275:30638-30643

38. **Pagano M** 2004 Control of DNA synthesis and mitosis by the Skp2-p27-Cdk1/2 axis. Mol Cell 14:414-416

39. **Payne SR, Zhang S, Tsuchiya K, Moser R, Gurley KE, Longton G, deBoer J, Kemp CJ** 2008 p27kip1 deficiency impairs G2/M arrest in response to DNA damage, leading to an increase in genetic instability. Mol Cell Biol 28:258-268

40. **Tanaka T, Akira S, Yoshida K, Umemoto M, Yoneda Y, Shirafuji N, Fujiwara H, Suematsu S, Yoshida N, Kishimoto T** 1995 Targeted disruption of the NF-IL6 gene discloses its essential role in bacteria killing and tumor cytotoxicity by macrophages. Cell 80:353-361

41. **Tanaka T, Yoshida N, Kishimoto T, Akira S** 1997 Defective adipocyte differentiation in mice lacking the C/EBPβ and /or C/EBPδ gene. EMBO J 16:7432-7443

42. **Zhu S, Yoon K, Sterneck E, Johnson PF, Smart RC** 2002 CCAAT/enhancer binding protein-beta is a mediator of keratinocyte survival and skin tumorigenesis involving oncogenic Ras signaling. Proceedings of the National Academy of Sciences of the United States of America 99:207-212

43. **Tang QQ, Otto TC, Lane MD** 2003 CCAAT/enhancer-binding protein beta is required for mitotic clonal expansion during adipogenesis. Proceedings of the National Academy of Sciences of the United States of America 100:850-855

44. **Bundy LM, Sealy L** 2003 CCAAT/enhancer binding protein beta (C/EBPbeta)-2 transforms normal mammary epithelial cells and induces epithelial to mesenchymal transition in culture. Oncogene 22:869-883

45. **Greenbaum LE, Li W, Cressman DE, Peng Y, Ciliberto G, Poli V, Taub R** 1998 CCAAT enhancer- binding protein beta is required for normal hepatocyte proliferation in mice after partial hepatectomy. J Clin Invest 102:996-1007

46. **Robinson GW, Johnson PF, Hennighausen L, Sterneck E** 1998 The C/EBPbeta transcription factor regulates epithelial cell proliferation and differentiation in the mammary gland. Genes & development 12:1907-1916

47. **Seagroves TN, Krnacik S, Raught B, Gay J, Burgess-Beusse B, Darlington GJ, Rosen JM** 1998 C/EBPbeta, but not C/EBPalpha, is essential for ductal morphogenesis, lobuloalveolar proliferation, and functional differentiation in the mouse mammary gland. Genes & development 12:1917-1928

48. **Glasser SR** 1990 Biochemical and structural changes in uterine endometrial cell types following natural or artificial deciduogenic stimuli. Trophoblast Research 4:377-416

49. **Harper JV, Brooks G** 2004 The mammalian cell cycle. Methods in Molecular Biology 296:113-153

50. **Brooks G** 2004 Cyclins, cyclin-dependent kinases, and cylcin-dependent kinase inhibitors Methods in Molecular Biology 296:291-298

51. **Wasner M, Haugwitz U, Reinhard W, Tschop K, Spiesbach K, Lorenz J, Mossner J, Engeland K** 2003 Three CCAAT-boxes and a single cell cycle genes homology region (CHR) are the major regulating sites for transcription from the human cyclin B2 promoter. Gene 312:225-237

52. **Bolognese F, Wasner M, Dohna CL, Gurtner A, Ronchi A, Muller H, Manni I, Mossner J, Piaggio G, Mantovani R, Engeland K** 1999 The cyclin B2 promoter depends on NF-Y, a trimer whose CCAAT-binding activity is cell-cycle regulated. Oncogene 18:1845-1853

53. **Brandeis M, Rosewell I, Carrington M, Crompton T, Jacobs MA, Kirk J, Gannon J, Hunt T** 1998 Cyclin B2-null mice develop normally and are fertile whereas cyclin B1-null mice die in utero. Proceedings of the National Academy of Sciences of the United States of America 95:4344-4349

54. **Vousden KH, Lu X** 2002 Live or let die: the cell's response to p53. Nat Rev Cancer 2:594-604

55. **Yoon K, Zhu S, Ewing SJ, Smart RC** 2007 Decreased survival of C/EBP beta-deficient keratinocytes is due to aberrant regulation of p53 levels and function. Oncogene 26:360-367

56. **Boggs K, Reisman D** 2007 C/EBPbeta participates in regulating transcription of the p53 gene in response to mitogen stimulation. The Journal of biological chemistry 282:7982-7990

57. **Schneider-Merck T, Pohnke Y, Kempf R, Christian M, Brosens JJ, Gellersen B** 2006 Physical interaction and mutual transrepression between CCAAT/enhancer-binding protein beta and the p53 tumor suppressor. The Journal of biological chemistry 281:269-278

58. **Grimm SL, Contreras A, Barcellos-Hoff MH, Rosen JM** 2005 Cell cycle defects contribute to a block in hormone-induced mammary gland proliferation in CCAAT/enhancer-binding protein (C/EBPbeta)-null mice. The Journal of biological chemistry 280:36301-36309

CHAPTER 3

The CCAAT/Enhancer Binding Protein Beta (C/EBPβ) is a Critical Regulator of Human Endometrial Stromal Proliferation and Decidualization

3.1 Abstract

The human endometrium is a dynamic tissue that shows remarkable changes in growth, differentiation and shedding during each menstrual cycle. The sequential actions of ovarian steroids estrogen (E) and progesterone (P) during the cycle induce a unique process known as pre-decidualization, which involves endometrial stromal proliferation, differentiation and remodeling. The molecular pathways underlying this hormonally induced cellular transformation event, which is a prerequisite for successful embryonic implantation, remain poorly understood. We have recently identified C/EBPβ, a CCAAT box-binding transcription factor, as a unique regulator of stromal proliferation and decidualization during implantation in mice. To reveal the role of this transcription factor in regulating human endometrial stromal cell (HESC) proliferation, we attenuated the expression of C/EBPβ via siRNA strategy in primary HESCs synchronized at G1/S boundary. Our results suggested that C/EBPβ controls the G1-S phase transition by regulating the expression of cyclin E and E2F1, which are critical regulators of DNA replication. Using a well-established *in vitro* system in which primary HESCs undergo decidualization upon addition of E, P, and a cyclic AMP analog, we have also addressed the role of C/EBPβ in stromal differentiation. Loss of function approaches, involving the use of C/EBPβ-specific siRNA or a dominant negative mutant of this transcription factor, blocked the decidualization process. In order to identify the molecular pathways regulated by C/EBPβ during decidualization, we performed gene expression profiling using RNA isolated from normal and C/EBPβ-deficient endometrial stromal cells. Our study revealed that several key regulators of stromal differentiation, such as BMP2 and Wnt4, IL-11Rα and STAT3, operate downstream of C/EBPβ during decidualization. Collectively, our studies uncovered a critical role for C/EBPβ in HESC proliferation and differentiation during decidualization.

3.2 Introduction

During each menstrual cycle, lasting about twenty-eight days, the human endometrium undergoes cyclical changes including proliferation, differentiation and menstruation, which are strictly controlled by the ovarian steroid hormones, estrogen (E) and progesterone (P), acting via their cognate receptors. The cycle first proceeds through an E-dominated proliferative phase (cycle days 1-14) associated with extensive epithelial and stromal regeneration and then enters a P-dominated secretory phase (cycle days 15-28). During the later phase, maturation and predecidualization of the stromal compartment occurs in preparation for embryo implantation (1;2). Predecidualization is a process that transforms fibroblast-like endometrial stromal cells into large, rounded, and secretory decidual cells. It is initiated in stromal cells surrounding the spiral arteries during the mid-late secretory stage of the cycle (2). If pregnancy ensues, a full-blown decidualization spreads throughout the stroma under the continued influence of E and P. The putative implantation window in the human opens for a short period of time in the mid secretory stage of the cycle (cycle days 20-24) (3). Abnormal decidualization of endometrial stroma is closely related with recurrent miscarriage and unexplained infertility in the human(4;5). A number of recent studies explored steroid hormone regulated molecular pathway that operate within the implantation window (6-10). However, the identities of the steroid-regulated pathways with defined functions during human endometrial stromal proliferation and decidualization process remain largely unknown.

Our previous studies identified C/EBPβ as a novel mediator of the biological actions of E and P during early pregnancy (11). This transcription factor belongs to a family of basic region/leucine zipper DNA-binding proteins (12), which regulate the transcription of target genes by binding to a conserved sequence, known as CCAAT box, in their promoter regions. Numerous studies have shown that C/EBPβ is a key regulator of proliferation and/or differentiation in multiple tissues, including the ovary, testis, liver, adipose tissue, immune system, skin cells and the mammary gland (13-22). Deletion of the C/EBPβ gene in the mouse led to female sterility, caused by the inability of the mutant mouse to form corpus luteum (13). We have found that the C/EBPβ-null uterus

exhibit a complete lack of decidual response to a mechanical stimulus, indicating that this transcription factor plays a critical role in regulating the hormone-mediated proliferation and differentiation program (11). Analyses of uteri of C/EBPβ-null mice indicated that loss of this transcription factor leads to an arrest in stromal cell cycle progression at the G2-M boundary. It was revealed that C/EBPβ controls the expression of multiple cell cycle regulatory molecules such as B-type cyclins B1 and B2, the phosphatase cdc25c and the cell cycle inhibitors p21, p27 and p53, all of which control cdk1 activity critical for G2-M transition.

In this study, we investigated the role of C/EBPβ in HESC proliferation and differentiation. In proliferating HESCs, we found that C/EBPβ controls the expression of mRNAs encoding several G1-S phase cell cycle molecules, such as cyclin E and E2F1. It also controls the activation of Cdk2 by governing the translocation of cyclin E into the nucleus. Lack of Cdk2 activity prevents the phosphorylation of Rb and the activation of E2F1, leading to a block in DNA replication. We have also used an *in vitro* stromal differentiation system to investigate the role of C/EBPβ in decidualization. Loss-of-function approaches, using a dominant-negative mutant of C/EBPβ or siRNA targeted against its mRNA, indicated that the differentiation process is strongly inhibited in the absence of this transcription factor. Using gene expression profiling, we uncovered several signaling pathways that operate downstream of C/EBPβ in HESCs during decidualization. These pathways included BMP2, Wnt4, IL-11Rα and STAT3, which were shown previously to be critical for decidualization in the mouse. C/EBPβ, therefore, controls HESC differentiation by regulating the expression of key signaling molecules that are conserved between the mouse and the human.

3.3 Materials and Methods

Primary HESC culture, synchronization and in vitro decidualization

The HESCs were provided by Dr. Robert Taylor (Emory University Medical School). These cells were isolated from biopsies taken from the proliferative stage endometria of normal cycling women and cultured in DMEM/F-12 (Invitrogen) containing 5% (v/v) fetal bovine serum (Hyclone), 50 µg/ml penicillin, and 50 µg/ml streptomycin (Invitrogen). Synchronization of HESCs in G1 phase was achieved by two consecutive rounds of thymidine block. Briefly, HESCs were cultured to 60% confluence in DMEM/F12-5% FBS and exposed to 2 mM thymidine for 12 h. Cells were then washed three times with PBS and the thymidine block was released by culturing the cells in fresh DMEM/F12-5% FBS for 12 h. The cells were subjected to a second round of thymidine block in the presence of DMEM/F12-5% FBS containing 2 mM thymidine for 12 h. Finally, the block was released by washing the cells with PBS, and placing them in fresh DMEM/F12-5% FBS. At this time (indicated as time zero), the cells were synchronized in G1 phase and poised to progress through the cell cycle in the presence of fresh medium. To induce *in vitro* decidualization, the cells were treated with medium, containing 1 µM progesterone (Sigma), 10 nM 17ß-estradiol (Sigma) and 0.5 mM 8-bromo-adenosine-3', 5'-cyclic monophosphate (8-Br-cAMP) (Sigma). The medium was changed every 48 h and the culture was maintained up to 10 days.

siRNA transfection

The HESCs were transfected with siRNA against C/EBPβ or scrambled siRNA as control (Ambion) following the manufacturer's protocol. Briefly, SilentFect transfection reagent (Bio-Rad Laboratories) was mixed with 40 nM of siRNA and added to HESCs at 80% confluency. After 24-48h, siRNA was removed and cells were treated with media containing E, P, and cAMP to induce decidualization. Cells were harvested at various time points following the hormone treatment. Gene expression was examined by quantitative real-time PCR using gene-specific primers.

Immunocytochemistry

HESCs were fixed in formalin solution (Sigma) at room temperature for 10 min followed by washing with PBS for 10 min. The cells were permeabilized by 0.25% Triton X-100 in PBS for 10 min and non-specific binding of antibodies was blocked with 10% donkey serum for 1 h at room temperature. Primary antibodies included anti-C/EBPβ (C-19, Santa Cruz Biotech), anti-cyclin D1 (Santa Cruz Biotech), anti-E2F1 (AbCam) , anti-Cyclin E (AbCam), anti-phospho-Rb (Ser-780, Santa Cruz Biotech), anti-phospho-Cdk2 (T-160, Abcam), and anti-STAT3 (C-20, Santa Cruz Biotech). Incubation with these antibodies was performed overnight at 4°C. Fluorescence (Cy3 or Delight 488)-conjugated anti-mouse IgG or anti-rabbit IgG (Jackson Lab) was used as secondary antibody. DAPI (1ug/ml in PBS) was used as counter staining.

Adenovirus-mediated expression of dominant negative C/EBPβ

An adenoviral vector expressing a dominant negative mutant of C/EBPβ (A-C/EBP) was provided by Dr. Charles Vinson (NCI/NIH). This vector expresses A-C/EBP under the control of CMV promoter. The protein is expressed with a HA epitope tag. An adenoviral vector lacking the A-C/EBP insert was used as a control. Control or A-C/EBP-carrying adenovirus was added to HESC monolayer (MOI: 10:1) at 80% confluence. After 24 h of viral transduction, the viral particles were removed and the cells were treated with the hormone cocktail to induce decidualization.

Quantitative real-time PCR analysis

Total RNA was extracted from cultured HESCs using the Trizol RNA purification kit (Invitrogen) according the manufacture's instructions. Reverse transcription was performed using the cDNA synthesis kit (Invitrogen) following the manufacture's instructions. The expression of C/EBPβ, Rb, E2F1, cyclin E, cdk2, PR, IGFBP-1, PRL, BMP2, Wnt4, STAT3, IL-11, and IL-11Rα mRNAs was examined by quantitative real-time PCR analysis using SYBR-Green super-mix (Bio-Rad Laboratories) and gene-specific primers. The gene expression level at any given time point or under a given

condition was quantified as fold change (mean±SD) relative to that at 0 h or control condition after normalization with respect to the internal control 36B4, a gene encoding a ribosomal protein.

Microarray Analysis

HESCs were obtained from individual biopsies taken from the proliferative stage endometria of three different normal cycling women and three separate primary cultures were established. These cultures were transfected with control (scrambled) siRNA or C/EBPβ-specific siRNA for 24 h and then treated with hormone cocktail (E+P+cAMP) with siRNA present for next 24 h. Then siRNAs were removed and incubation with hormone cocktail was continued for another 48 h. Cells were harvested and total RNA was prepared by TRIzol ® Reagent (Invitrogen, CA). RNA integrity was verified using Agilent 2100 bioanalyser (Agilent Technologies Inc., Santa Clara, CA, USA) at the Biotechnology Center of the University of Illinois, Urbana and Champaign. All RNA samples showed an RNA integrity number (RIN) greater than 9. Each RNA sample was processed for microarray hybridization using GeneChip® Human Geneme U133 A Plus 2.0 arrays (Affymetrix Inc., Santa Clara, CA), following the established protocol.

Quality Control (QC) assessment, data processing and statistical analysis were done in R, a software for statistical computing and graphics, using packages from the Bioconductor project as indicated. QC assessment showed that all arrays were of acceptable quality. The raw, probe-level data were processed with the GCRMA algorithm, which performs a GC-based background-correction, does a normalization between arrays and summarizes the multiple probes into one probe set value using a median polish algorithm. Probe sets that were not called “present” on at least one array or “marginal” on at least two arrays using Affymetrix’s Call Detection Algorithm, and did not have at least one array with a GCRMA value greater than 2.5, were considered not expressed in all samples and were filtered from further analysis. 13,351 probe sets passed these filters and were tested for differential expression. The False Discovery Rate method[8] was used to adjust the p-values, and FDR p-values < 0.05 were considered

significantly different. The significantly regulated genes were further sorted by gene ontology and pathways using PANTHER Classification Software.

Statistical Analysis

The RNA and protein samples were prepared from at least three separate primary cultures subjected to the same experimental treatment. The real-time PCR results are expressed as mean ± S.D of three separate measurements. Statistical significance of the data was determined using one-way ANOVA followed by student t-test. A p-value of <0.05 was considered significant.

3.4 Results

C/EBPβ-deficient HESCs exhibit impaired proliferation

During the proliferative phase of the menstrual cycle, HESCs undergo repeated cell division to rebuild the *functionalis* layer of the endometrium. This proliferative activity can be recapitulated in an *in vitro* primary culture system in which the undifferentiated HESCs isolated from proliferative phase of the cycle undergo many rounds of cell division when maintained in the presence of serum. We observed that when HESCs cultured in a medium containing low (1%) serum are placed in medium containing high (5%) serum, there was a significant increase in the rate of proliferation of these cells. We observed that the level of C/EBPβ mRNA was markedly elevated within 12 h of serum addition and the increased expression of this factor was maintained as the cells continued to proliferate (Fig. 3.1A).

To investigate the role of C/EBPβ in HESCs proliferation, we employed a siRNA strategy to suppress its mRNA expression. As shown in Figure 1B, we observed that transfection of HESCs with C/EBPβ-specific siRNA reduced its mRNA expression ~70% within 24 h of treatment. At 48 h following siRNA transfection, there was greater than 85% reduction in C/EBPβ mRNA levels. Transfection of control (scrambled) siRNA had no appreciable effect on C/EBPβ mRNA levels (Fig.3.1B). Immunocytochemical

analysis (Fig. 3.1C) confirmed the drastic reduction in the level of C/EBPβ protein upon treatment with C/EBPβ-specific siRNA.

Our primary cultures consisted of a heterogeneous population of proliferating HESCs at various stages of the cell cycle. To synchronize the growth of these cells, we subjected them to two consecutive rounds of thymidine block, a well-established method of cell synchronization (23). It was shown previously that mammalian cell cycle is arrested at the G1 phase when cells are switched to a thymidine-rich (2mM) medium. The excess thymidine causes a nucleotide imbalance in the cell, thereby blocking the DNA replication and arresting the cell cycle at the late G1 phase prior to its entry into the S phase (23). The cells that escaped the first round of thymidine block were subjected to a second round of thymidine treatment. To ascertain the efficiency of cell synchronization, we monitored BrdU incorporation in these cells before and after the release of the block. We added the BrdU to the culture 1 h before the release of 2^{nd} thymidine block or at the time of the release of the thymidine block.

We assessed the BrdU incorporation at different time points by immunocytochemistry using anti-BrdU antibody (Fig. 3.2A). No BrdU incorporation was detected when it was added prior to the release of the thymidine block, confirming a cell cycle arrest prior to the entry into the S phase. When BrdU was added at the time of the release of the block, a large number (~70%) of cells entered S phase and exhibited substantial BrdU incorporation by 4 h (Fig. 3.2A and 3.2B). By 8 h, more than 80% of the cells entered S phase. Collectively, these results indicated the HESCs could be effectively synchronized by double thymidine block at G1 –S phase boundary and the S phase entry can be easily induced by removing the extra thymidine from the culture.

We next attenuated the expression of C/EBPβ in synchronized HESCs using siRNA targeted to its mRNA. The siRNA was added during the second round of the thymidine block and the transfection was carried out for 24 h. The siRNAs were then removed and the cells were released from thymidine block to resume their proliferation. To monitor the time course of DNA synthesis, BrdU was added to the cultures and its incorporation in normal and C/EBPβ-deficient HESCs was monitored by

immunocytochemistry (Fig. 3.2C). We observed that the BrdU incorporation was markedly reduced in C/EBPβ-deficient HESCs relative to the control cells at 4, 7 and 10 h following the release of the G1-S arrest (Fig. 3.2C and 3.2D). These results suggested that the downregulation of C/EBPβ impairs the entry and progression of HESCs into the S phase.

To further examine the role of C/EBPβ in HESC proliferation, we examined the mitotic activity of these cells, using an antibody that recognizes phosphorylated Ser 10 of histone 3 (P-His3), a unique mitotic phase marker(24). In proliferating animal cells, the P-His3 appears in pericentromeric heterochromatin in late G2 interphase cells and spreads throughout the condensing chromatin as mitosis progresses. We observed that the control HESCs displayed specific staining of P-His3 by 13 h following the release of thymidine block (Fig. 3.3A and 3.3B). The number of P-His-positive cells was increased at 16.5 h and the positive staining sustained until 18 h. In contrast, the P-His3 staining was drastically reduced in C/EBPβ-deficient HESCs (Fig. 3.3A and 3.3B). These results indicated that the majority of the C/EBPβ-deficient HESCs did not undergo the G2-M transition, which is presumably a consequence of their G1-S arrest.

C/EBPβ regulates the expression and nuclear localization of cyclin E and activated phosphorylation of cyclin E-dependent kinase (cdk2) in proliferating HESC

We next investigated whether C/EBPβ controls the G1-S transition of proliferating HESCs by regulating the expression and activation of cyclins and cyclin-dependent kinases (cdks) that specifically control this checkpoint. We monitored the expression of mRNAs corresponding to cyclins D1, E and A, and cdks 2 and 4, which are hallmarks of G1 and S phases of the cell cycle. Immunocytochemical studies showed comparable cyclin D1 protein expression in HESCs in the presence or absence of C/EBPβ (Fig. 3.4A). When we examined the expression of cyclin E in control siRNA-treated cells, we observed that the mRNA encoding this cyclin is maximally expressed at 4 h following the release of the thymidine block and its level decreased to basal level by 8 h. The expression of cyclin E mRNA was markedly reduced in C/EBPβ siRNA-treated cells (Fig. 3.4B). No significant alteration was noted in the expression of cyclin A mRNA

(Fig. 3.4D). When we monitored cyclin E protein level via immunflorescence, we noted robust nuclear staining of this protein in control cells that have entered the S phase (Fig. 3.4C). In contrast, in C/EBPβ-deficient cells, the intensity of the nuclear staining of cyclin E was strikingly reduced, while more cytoplasm staining was observed in cells containing normal levels of C/EBPβ. This altered localization of cyclin E in the C/EBPβ-deficient cells presumably results from the failure of this cyclin to translocate from nucleus to cytoplasm and is likely to contribute to the block in G1-S transition of these cells.

The G1-S arrest could also be a consequence of the loss of activity of cdk2 and cdk4, which form active cyclin-cdk complexes with E-type and D-type cyclins, respectively. No statistically significant alteration in the expression levels of cdk4 and cdk2 mRNAs was observed C/EBPβ-deficient cells compared to the control cells (Fig. 3.4E and 3.4F), Since we found the expression and nuclear localization of cyclin E is greatly reduced in the absence of C/EBPβ, we examined the activation state of cdk2 in these cells. Cdk2 activity requires the recruitment of cyclin E, which triggers a phosphorylation at threonine 160 in its activation loop (25). Using an antibody that specifically recognizes T160 phosphorylation, we discovered that this activating modification of cdk2 was markedly reduced in C/EBPβ-deficient cells compared to control cells (Fig. 3.4G). The lack of a functional cyclin E-cdk2 complex likely contributes to the G1-S arrest of C/EBPβ-deficient cells.

C/EBPβ -mediated pathways control the phosphorylation of Rb and the expression of E2F1 in HESC

The retinoblastoma protein (Rb) is an important inhibitor of cell cycle progression. Phosphorylation is the key post-transcriptional modulation controlling its function (26). In its hypophosphorylated active form, Rb binds to the E2F family of transcription factors, resulting in suppression of their transcriptional activity. In G1 phase, cyclin D/cdk4 and cyclin E/cdk2 sequentially phosphorylate Rb at T798 and T780, respectively(27;28). These modifications produce a hyperphosphorylated Rb, allowing its disassociation from E2Fs. This event allows the activation of E2Fs, which then regulate

downstream target genes required for entry into S phase (29). To assess whether Rb is involved in the G1-S arrest of C/EBPβ-deficient HESCs, we examined its expression level and phosphorylation status. We found that the level of Rb mRNA was not significantly altered in control- and C/EBPβ siRNA-treated HESCs (Fig. 3.5A). Next, we employed an antibody that specifically recognizes the cyclinE/cdk2 phosphorylation site of Rb to examine its phosphorylation status. (27;28) Immuncytochemical analysis revealed that the phosphorylation of Rb at T780 is markedly reduced in C/EBPβ-deficient HESCs (Fig. 3.5B), consistent with the observed lack of cyclin E/cdk2 activity in these cells. This finding suggests that hypophosphorylated Rb will remain bound to E2F and inhibit its transcriptional activation, which is critical for G1-S transition of the HESCs.

We also examined whether the expression of the E2F family members is controlled by C/EBPβ in HESCs. We noted that the mRNA corresponding to E2F1 is prominently expressed in HESCs during the cell cycle (Fig. 3.5C). In contrast, the levels of E2F2 and E2F3 mRNAs were relatively low (data not shown). As shown in Figure 5C, the level of E2F1 mRNA rose rapidly as the HESCs entered the S phase when the cell proliferation is resumed at the end of the thymidine block. This induction in E2F1 gene expression was absent in C/EBPβ-deficient cells. This reduction in E2F1 expression was further confirmed by examining E2F1 protein levels in control and C/EBPβ-deficient HESCs by immunocytochemistry (Fig. 3.5D).

In vitro decidualization of HESCs; dissection of the proliferation and differentiation events

Our laboratory and others have previously described an *in vitro* decidualization system using primary cultures of HESCs, isolated from proliferative phase human endometrium (9;30-33). Addition of a hormone cocktail containing E, P, and a cAMP analog to these cultures initiates differentiation of these cells. As shown in Fig. 3.6A, within 3-6 days of hormone treatment, the fibroblastic HESCs undergo a striking morphological change into plump, epitheloid, decidual cells. This morphological transformation was associated with appropriate biochemical changes characteristic of decidualization. The expression of mRNAs corresponding to several well-characterized

decidual markers, such as PRL, IGFBP-1, and IL-11 was increased within 2-4 days of culture and reached a peak by days 6-8 (Fig. 3.6B).

Since stromal proliferation always precedes stromal differentiation during decidualization, it raises an important question: are the proliferation and differentiation events connected and interdependent? To address this issue, we first tested whether the HESCs are still able to proliferate when the hormone cocktail is added to the cultures to initiate differentiation. When we compared the rate of proliferation of HESCs in the primary cultures, we found that the cells, which continued to divide and grow robustly in the absence of the hormone cocktail, failed to proliferate significantly in the presence of the cocktail (Fig. 3.6C). Therefore, it is clear that the initiation of differentiation of the stromal cells is accompanied by a cessation of their proliferative activity.

We next asked whether the proliferation of HESCs is a necessary prerequisite for their differentiation. To analyze this issue, we first arrested HESC proliferation by double thymidine block and synchronized these cells at G1 phase (Fig. 3.6D). We then added hormone cocktail to initiate decidualization of these cells with or without releasing the cell cycle block. When the block was released, treatment with hormone cocktail for 72 h induced differentiation as evidenced by the enhanced expression of the well known decidualization markers: PRL, IGFBP-1 and IL-11. The magnitude of expression of these biomarkers was comparable to that seen in an asynchronous culture upon addition of hormone cocktail (Fig. 3.6D). Interestingly, we observed that the HESCs, still arrested in cell proliferation in the presence of the thymidine block, exhibited a similar robust induction of the decidualization markers (Fig. 3.6D), indicating that a block in cell proliferation did not prevent stromal decidualization. These results established that we have a unique primary culture system in which the differentiation of HESCs can be studied independent of cell proliferation.

C/EBPβ is an essential regulator of HESC differentiation

To assess the functional role of C/EBPβ in HESC differentiation, we began by monitoring the expression of this factor in the *in vitro* decidualization system. We observed that the expression of C/EBPβ mRNA was significantly enhanced within 12-24

h of treatment with hormone cocktail (Fig. 3.7A). This elevated expression of C/EBPβ mRNA continued at 48 h. It declined at 72 h but was maintained at a significant level up to 9 days of culture. The expression of C/EBPβ protein, monitored by immunocytochemistry, also showed significant enhancement at 48 h following treatment with hormonal cocktail (Fig. 3.7B). Since the enhanced expression of C/EBPβ occurred early (12-24 h) during the decidual program, it raised the possibility that this transcription factor is a critical early regulator of this process.

To test this possibility, we employed a dominant negative mutant to inhibit its transcriptional activity and examined the consequences of this intervention on well-defined differentiation end points. In this mutant, termed A-C/EBP, the basic region critical for DNA binding is replaced by acidic amino acids and the transactivation domain is deleted producing a molecule that forms stable heterodimers with the leucine zipper region of C/EBPβ (34). Expression of A-C/EBP in a cell inhibits endogenous WT C/EBPβ by blocking its DNA binding activity and thereby interrupting its transcriptional function (34;35). A-C/EBP inhibits the DNA binding of all C/EBP family members but not other B-ZIP transcription factors (36). We noted that C/EBPβ is the predominant C/EBP family protein that is expressed in HESCs during decidualization (Fig. 3.7C), (37). While a robust signal for C/EBPβ mRNA is seen in these cells, very little C/EBPα or δ mRNA is detectable during decidualization. These observations suggested that the inhibitory action of A-C/EBP in the primary stromal cultures is directed primarily at C/EBPβ.

The expression of A-C/EBP in the adenovirus-transduced HESCs was first ascertained by immunocytochemistry using an anti-HA antibody that recognizes the HA-tagged dominant negative mutant (Fig. 3.7D). A viral vector without the A-C/EBP insert was used as a control. Within 24 h of viral transduction, >90% of human stromal cells were found to express A-C/EBP. 24 h after virus addition, the cells were treated with the hormone cocktail and the culture was continued for 72 h. At that time point, total RNA was extracted from cells transduced with control or A-C/EBP virus and expression of well-known decidualization markers was monitored. Our results indicated that A-C/EBP drastically reduced expressions of PRL, IGFBP-1 and IL-11 mRNAs (Fig. 3.7E). The A-

C/EBP adenovirus addition had no significant effect on the expression levels of either C/EBPβ or PR mRNA (Fig. 3.7E). These results demonstrated that the dominant-negative mutant repressed human decidualization and it achieved this presumably by blocking the transcriptional activity of the endogenous C/EBPβ.

To further confirm the functional role of C/EBPβ in human stromal differentiation, we employed siRNA strategy to suppress the C/EBPβ mRNA expression and then examined its effect on decidualization process by monitoring the expression of well-known decidual markers. As shown in Fig. 3.1B and 3.1C, C/EBPβ-specific siRNA efficiently reduced the expression of C/EBPβ, both at mRNA and protein level, in HESCs. When we investigated the functional consequences of this blockade of C/EBPβ expression during stromal differentiation, we observed that siRNA-mediated down-regulation of C/EBPβ in the HESCs led to a significant reduction in expression of PRL, IGFBP-1 and IL-11. In contrast, the level of PR remained mostly unaltered in cells treated with either C/EBPβ or control siRNA (Fig. 3.7F). Collectively, these results revealed an essential role of C/EBPβ in the stromal differentiation process.

Identification of the C/EBPβ-regulated gene networks that control human endometrial stromal decidualization

Since C/EBPβ critically controls the differentiation of HESCs in primary cultures, we sought to explore the downstream pathways of this transcription factor. Our approach involved attenuation of C/EBPβ expression by C/EBPβ-specific siRNA followed by gene expression profiling using Affymetrix human GeneChip arrays to identify its downstream target genes. Three independent primary HESC cultures were established from biopsies derived from three different individuals, and subjected to *in vitro* decidualization in the presence control or C/EBPβ-specific siRNA. Total RNA isolated from these cells was used for three sets of microarray analyses. Our study identified many genes whose expression was significantly altered in response to C/EBPβ-specific siRNA in all three arrays. The expression of 1270 genes was up-regulated and that of 1180 genes was down-regulated (>1.5 –fold, p-value <0.1) in response to the siRNA intervention. The candidate C/EBPβ-regulated genes in the human stromal cells were classified according to their

known biological functions by Panther Classification System (Tables 1 and 2). These pathways represented a variety of different biological categories, such as regulators of proliferation, differentiation, ECM and cytoskeleton structure, inflammation regulation and immune function, which are all critical for implantation.

BMP2/Wnt4 and IL-11 signaling pathways are downstream targets of regulation by C/EBPβ during HESC differentiation

Among the many genes whose expression was down regulated in response to the attenuation of C/EBPβ expression, several biological categories were prominent because of their known roles in decidualization (Table 1). One of them represented the TGF beta family signaling pathway involving the morphogen BMP2, which is an essential regulator of decidualization in both mouse and human (9;38). Another category that stood out was the Wnt signaling pathways such as Wnt4. It is of interest to note that we have demonstrated that Wnt4 is a downstream target of BMP2 in both mouse and human endometrial stromal cells during decidualization (9). One can, therefore, envision a linear pathway involving C/EBPβ, BMP2 and Wnt4, operating during HESC differentiation. The other category of C/EBPβ target genes included genes encoding components of the IL-11 cytokine pathway, such as IL-11 receptor α and the signal transducer STAT3, which are important regulators of decidualization (39-42).

We confirmed the results of our microarray analysis by performing quantitative real-time PCR analysis, using RNA isolated from the HESCs treated with C/EBPβ-specific or control siRNA. We observed that siRNA targeted to C/EBPβ mRNA significantly inhibited the expression of BMP2, Wnt4, IL-11Rα and STAT3 as well as that of the classical decidualization markers PRL and IGFBP1 (Fig. 3.8). The identification of BMP2, Wnt4, IL-11R α and STAT3, which are known regulators of decidualization, as downstream targets of regulation by C/EBPβ supports the central role played by this transcription factor in HESC differentiation.

3.5 Discussion

It was shown previously by our laboratory that C/EBPβ mediates steroid-regulated stromal proliferation and differentiation during implantation in the mouse (11). The present study was undertaken to address the role of C/EBPβ in human endometrial stromal decidualization. In human endometrium, stromal proliferation occurs mainly in the proliferative phase, which precedes stromal differentiation, known as predecidualization (43). The post-ovulatory increase of P elevates the intercellular cAMP in stromal cells, thereby activating the protein kinase A signaling pathway, which contributes to the stromal differentiation process (44). Several factors are known to play important roles in controlling human endometrium proliferation or differentiation (6-10). Our recent studies described C/EBPβ expression in both epithelial and stromal compartments during proliferative and secretory phases of the menstrual cycle (45). An intense nuclear expression of C/EBPβ in glandular epithelium and differentiating stroma of human endometrium during the mid secretory phase of the cycle (45). These findings raised the possibility that C/EBPβ-regulated pathways control HESC function during various stages of the menstrual cycle. In the present study, we employed primary cultures of endometrial stromal cells isolated from biopsies collected at the proliferative stage of the menstrual cycle to demonstrate that C/EBPβ controls HESC proliferation as well as differentiation via its downstream signaling pathways.

Primary HESCs can either proliferate or undergo differentiation, depending on the culture conditions. Several laboratories have shown that the HESCs can be maintained in proliferating state for many generations or induced to undergo differentiation in response to a hormonal cocktail containing P, E, and a cAMP analog (30;43;46-48). This primary culture system, therefore, presents a unique opportunity to explore the role of C/EBPβ in HESCs proliferation and differentiation. We observed that C/EBPβ expression is markedly induced concomitant with stimulation of HESC proliferation by high serum. A loss-of-function approach using siRNAs showed that C/EBPβ is a key regulator of the G1-S transition during proliferation of HESCs.

The mammalian cell cycle proceeds through distinct stages: G1, S, G2, and M (49). Progression through each phase of the cell cycle is under the strict control of various cyclins, cyclin-dependent kinases (cdks), and other cycle regulatory molecules (49-52) The cyclical changes in the expression and localization of cyclins and the sequential activation of specific cyclin/cdk complexes drive the cell cycle from one phase to another (50). The formation and activation of the cyclin D-cdk4/6 and cyclin E-cdk2 complexes are critical for G1-S transition. These complexes participate in the phosphorylation of Rb, which critically controls cell cycle progression via its inhibitory association with the E2F family of transcription factors (27;28). Hyperphosphorylated Rb dissociates from E2Fs, allowing the activation of these transcription factors (26;27;29;53;54). Any interruption in the chain of events starting from the activation of D-type or E-type cyclins and their target kinases to the eventual activation of E2Fs would result in a G1-S phase block. Our study showed that down regulation of C/EBPβ expression in synchronized HESCs inhibits the expression of cyclin E and impairs its nuclear accumulation. This in turn prevents cyclin E-dependent phosphorylation and activation of cdk2 function. Lack of cdk2 activity prevents phosphorylation on Rb and its dissociation from E2Fs. Additionally, E2F1 mRNA and protein expression is diminished in C/EBPβ-deficient HESCs. The combination of decreased production of E2F1 and a block in its transcriptional activation due to a failure to dissociate from Rb likely contributes to the G1-S arrest in the C/EBPβ-deficient cells.

Recent studies in our laboratory, using a C/EBP-null mouse model, showed that despite the loss of C/EBPβ expression, uterine stromal cells are able to enter the S phase of the cell cycle and execute DNA synthesis in response to a decidual stimulation (Wei Wang, I. C. Bagchi and M. K. Bagchi, unpublished results). The loss of this transcription factor did not affect the expression or activity of D-type, A-type or E-type cyclins or their target kinases cdk2 and cdk4, which control G1-S transition of uterine stromal cells. The C/EBP-null stromal cells, however, were arrested at the G2-M checkpoint of the cell cycle. Further analysis revealed that C/EBPβ regulates the expression of several key molecules that control G2-M transition. These included cyclins B1 and B2, the phosphatase cdc25c, which controls cdk1 activity, and the cell cycle inhibitors p21, p27,

p53. These findings are in sharp contrast to our current results in the HESCs that C/EBPβ is a key regulator of S phase entry and progression. It is, therefore, evident that the C/EBPβ-regulated mechanisms guiding controlling proliferation is not conserved in the mouse and the human.

HESCs undergo limited decidualization, known as predecidualization, during the secretory phase of the menstrual cycle (1;2). This unique cellular transformation is an essential prerequisite for implantation. In a recent study, we observed intense expression of C/EBPβ in the luminal, glandular and stromal cells of the secretory phase endometrium (45). A marked increase in nuclear C/EBPβ protein immunostaining was seen in stromal cells beginning about cycle day 21, coincident with the start of endometrial receptivity. This result is consistent with previous reports, which described that C/EBPβ regulates the expression of decidual prolactin, a major marker of decidualization of human stromal cells (37;55). In the present study, we analyzed the expression of C/EBPβ in a well established *in vitro* stromal primary culture system in which decidualization can be efficiently induced by a hormonal cocktail containing E, P, and a cAMP analog (48). The enhanced expression of C/EBPβ occurred early during the decidual program, consistent with the hypothesis that it regulates downstream pathways critical for this process. Indeed, when we attenuated the transcriptional activity of C/EBPβ using a dominant-negative mutant or suppressed its expression by administering siRNA targeting its mRNA, the differentiation process was strongly inhibited, providing strong evidence that C/EBPβ plays an essential role in human stromal cell decidualization.

Although C/EBPβ is a major regulator of uterine functions in the mouse and the human, little is known about its target genes in this tissue. We explored the *in vitro* decidualization system to identify C/EBPβ-regulated downstream pathways during stromal differentiation. Using gene expression profiling, we identified as many as 2350 known genes as potential targets of regulation (both up and down regulated) by C/EBPβ during this differentiation process. These genes encoded molecules with a wide range of biological functions such as cellular proliferation and differentiation, cellular signaling,

regulation of cytoskeleton structure, inflammation and immune regulation, enzyme metabolism, transport and ion channel, etc. The identification of large number of molecular pathways downstream of C/EBPβ is not unexpected, given the complexity of the decidualization process, which involves biological phenomena as varied as differentiation, vascular remodeling and maternal immunosuppression. A remarkable finding of this study is the discovery that C/EBPβ is the potential regulator of the BMP2-Wnt4 pathway, which is intimately related with the formation, regulation and function of the decidual tissue (9;38). Previous studies established that mice deficient in uterine BMP2 are infertile and exhibit a severe defect in stromal differentiation (38). We identified Wnt 4 as a downstream target of BMP2 regulation in stromal cells undergoing decidualization (9). Attenuation of Wnt 4 expression by siRNAs greatly reduced stromal differentiation *in vitro*, suggesting that it is a candidate mediator of BMP2-induced decidualization. It is, therefore, reasonable to conclude that BMP2/Wnt4 pathway represents a major avenue via which C/EBPβ exerts its regulatory effects on human stromal decidualization.

C/EBPβ is long known as an acute phase protein in the liver and as a regulator of several genes involved in the regulation of inflammatory responses (56). A role for C/EBPβ as an inflammation regulator has not been explored in the context of a reproductive tissue. The present study revealed that C/EBPβ is a major regulator of signaling by the IL-11 cytokine, which is generally anti-inflammatory, during decidualization. IL-11 signals via a cell surface receptor complex comprising IL11 receptor a and the common signaling component gp130, which act through the JAK/STAT pathway (57). A number of studies have shown that IL-11 signaling pathway is essential for decidualization. In mice, disruption of IL-11Ra expression leads to decidualization failure and infertility (39;41). In human endometrium, IL-11 and IL11Ra are expressed in decidualizing stromal cells of mid-late secretory-phase, suggesting a possible role in this process (58). In human primary stromal cultures, addition of IL-11 advanced P induced decidualization (59). *In vivo* immunostaining showed that this cytokine is significantly induced in the decidualizing stromal cells in the late secretory endometrium (58;60). In recent studies, dysregulation of IL-11-IL11Ra signaling

pathway was linked to unexplained female infertility, presumably due to defects in decidualization (61-63). Our microarray analysis showed that IL11Ra mRNA expression is downstream of C/EBPβ, establishing an important functional link between this transcription factor and a local cytokine signal pathway, which is important for biological processes leading to the successful establishment of pregnancy.

In summary, the present study demonstrated that the transcription factor-C/EBPβ controls the proliferation and differentiation of HESCs by regulating various target genes. This work also indicated that several key regulators of stromal differentiation, such as BMP2 and Wnt4, and IL-11Rα and STAT3, operate downstream of C/EBPβ during decidualization. Future studies will investigate the mechanisms that govern the regulation of these downstream pathways by C/EBPβ.

3.6 Acknowledgements

The human endometrial stromal cells were provided by Dr. Robert Taylor of Emory University Medical School, Atlanta, GA. The adenovirus containing dominant-negative A-C/EBP was kindly provided by Dr. Charles Vinson of NCI/NIH. This work was supported by the Eunice Kennedy Shriver NICHD/NIH through cooperative agreement U54 HD055787 as part of the Specialized Cooperative Centers Program in Reproduction and Infertility Research.

3.7 Figures and legends

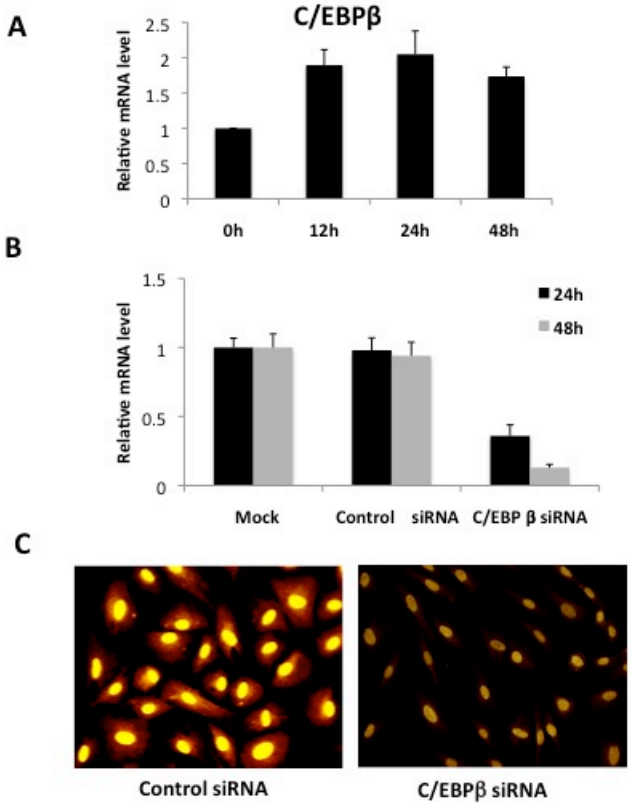

Figure 3.1 Down regulation of C/EBPβ in HESCs

Primary cultures of HESCs were grown in DMEM/F-12 (1:1) medium containing 1% charcoal-stripped FBS for two days.

A. The cells were harvested at different times after increasing the serum concentration in medium to 5% FBS. Total RNA was isolated and subjected to real time PCR using gene-specific primers for C/EBPβ.

B. HESCs were transfected with siRNA (40nM) targeted against C/EBPβ or control siRNA (scrambled) as described in the Materials and Method. Total RNA was prepared from HESCs at 24 and 48 h after transfection with control or C/EBPβ siRNA and subjected to real time PCR using C/EBPβ-specific primers.

Fig. 3.1 (continued) Down regulation of C/EBPβ in HESCs

C. The expression of C/EBPβ protein was monitored at 72 h after siRNA transfection using immunocytochemistry.

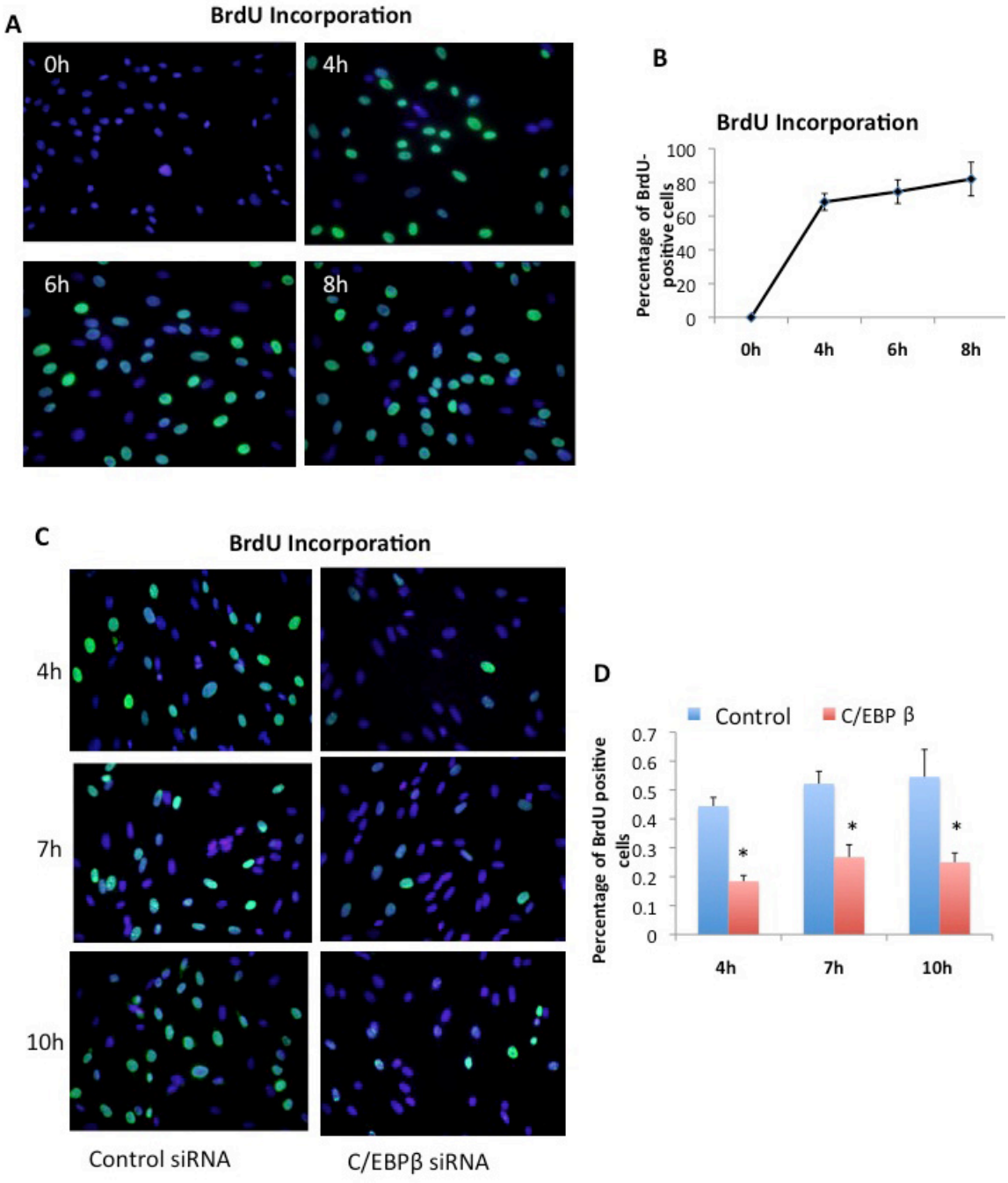

Figure 3.2 Blockade of G1-S transition in C/EBPβ-deficient HESCs

A. HESCs were seeded in slide chambers, and synchronized in the G1 phase using a double thymidine block as described in the Material and Methods. BrdU was added 1 h before or at the time of the second thymidine addition. The cells were fixed in formalin

Fig. 3.2 (continued) Blockade of G1-S transition in C/EBPβ-deficient HESCs

solution at different times after the second thymidine addition, and then subjected to immunocytochemistry using a BrdU antibody.

B. The percentage of BrdU-positive cells was quantitated at different times after the thymidine treatment. Total cell number was estimated by DAPI nuclear staining. Data were expressed as average +/- S.D of 5 separate measurements.

C. HESCs were seeded on slide chambers, synchronized using a double thymidine block, and then transfected with siRNA (40nM) targeted against C/EBPβ or control siRNA for 36 h with thymidine present in the culture. After transfection, cells were washed and placed in fresh 5% DMEM/F12 with BrdU added to the culture. Cells were fixed at different times after BrdU addition and then subjected to immunocytochemistry using anti-BrdU antibody.

D. The percentage of BrdU-positive cells was quantitated in HESC cultures treated with control or C/EBPβ siRNA at different times after BrdU addition. Total cell number was estimated by DAPI nuclear staining. Data were expressed as average +/- S.D of five separate measurements. * indicates statistically significant change ($P<0.05$) when compared with control siRNA treatment.

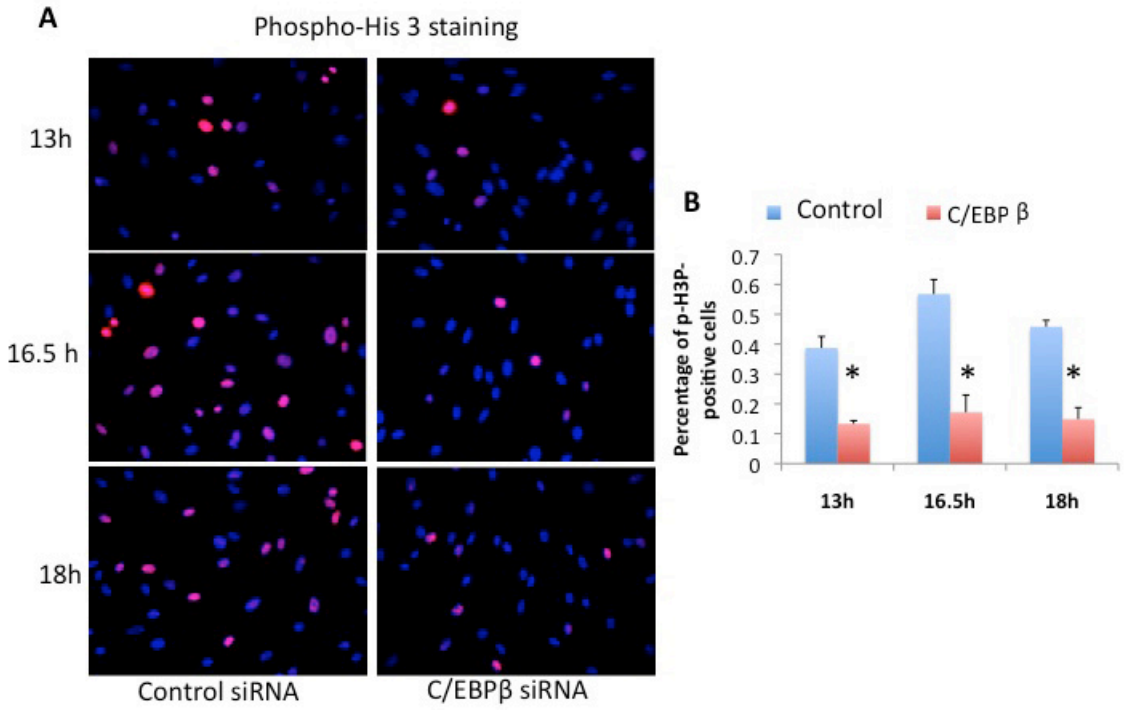

Figure 3.3 Examination of G2-M phase transition in C/EBPβ-deficient HESCs

HESCs were seeded on slide chambers, synchronized by double thymidine block, and then transfected with siRNA (40nM) targeted against C/EBPβ or control siRNA (scrambled) for 36 h with thymidine block in effect.

A. After siRNA transfection, cells were washed and cultured in fresh 5% FBS DMEM/F12. Cells were fixed at different times and then subjected to immunocytochemistry using an anti-phospho-His 3 antibody.

B. The percentage of phospho-His 3-positive cells was quantitated in HESC cultures treated with control or C/EBPβ siRNA at different times after release of the thymidine block. Total cell number was estimated by DAPI nuclear staining. Data were expressed as average +/- S.D of five separate measurements. * indicates statistically significant change ($P<0.05$) when compared with control siRNA treatment.

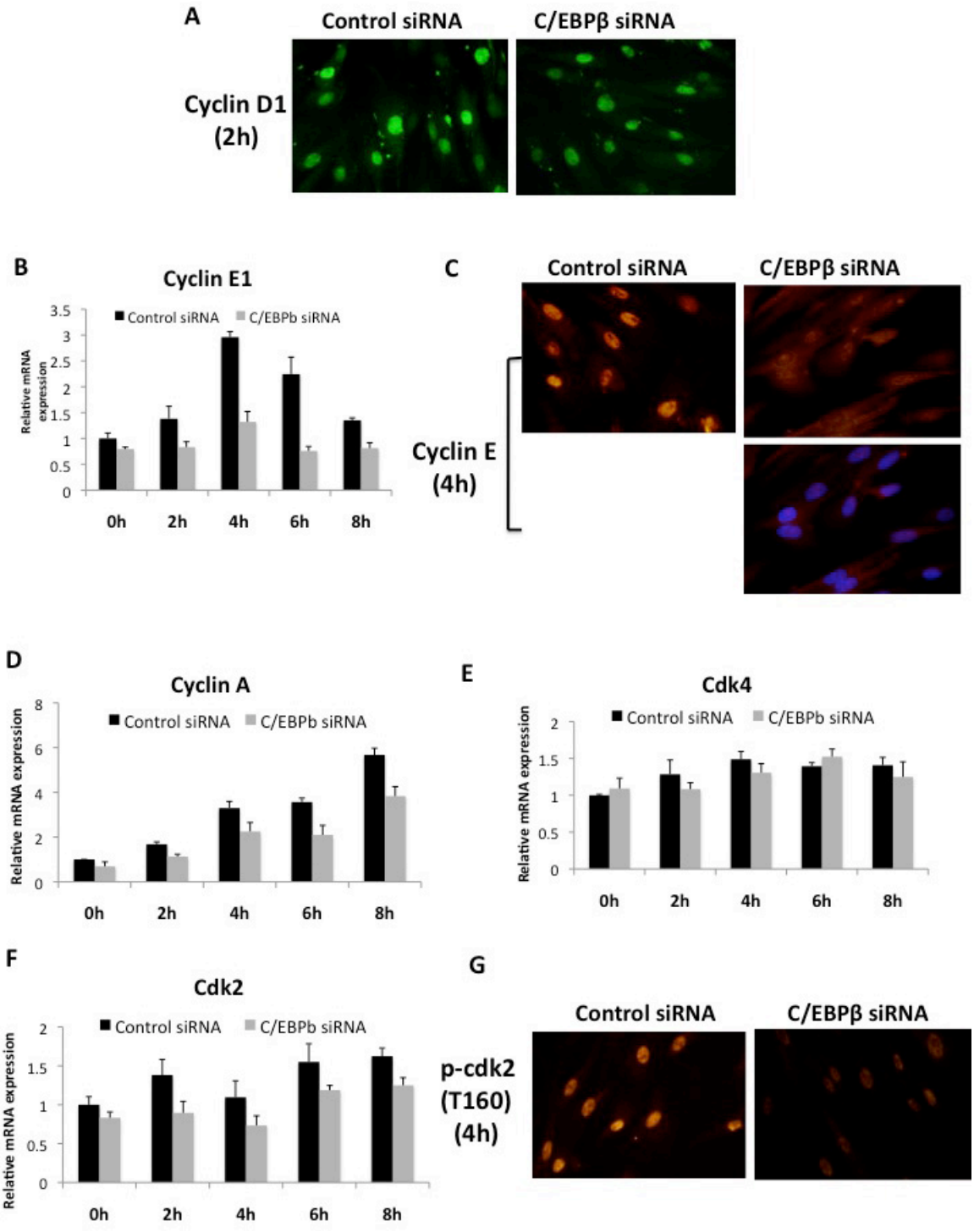

Figure 3.4 Analysis of expression of G1-S phase regulators in C/EBPβ-deficient HESCs

Fig. 3.4 (continued) Analysis of expression of G1-S phase regulators in C/EBPβ-deficient HESCs

HESCs were synchronized at G1 phase by double thymidine block and then transfected with siRNA (40nM) targeted against C/EBPβ or control siRNA (scrambled) for 36 h with thymidine present in the culture. Following transfection, cells were washed and placed in fresh medium containing 5% FBS and then harvested at different times. The cells were fixed and subjected to immunocytochemistry using antibodies against cyclin D1 (A), cyclin E (C), and activated form of cdk2 (G). Total RNA was also isolated from these cells and the expression of mRNAs corresponding to cyclin E (B), cyclin A (D), Cdk4 (E), and Cdk2 (F) were monitored by real time PCR, using gene-specific primers. 36B4 was used as an internal control. The relative expression of these genes at different times were determined by normalizing with respect to the level in control siRNA-treated cells at 0 h. The values represent mean ± S.D of three separate measurements. * indicates statistically significant change ($P<0.05$) when compared with control siRNA treatment. Control siRNA treated: black tower and C/EBPβ siRNA treated: grey tower.

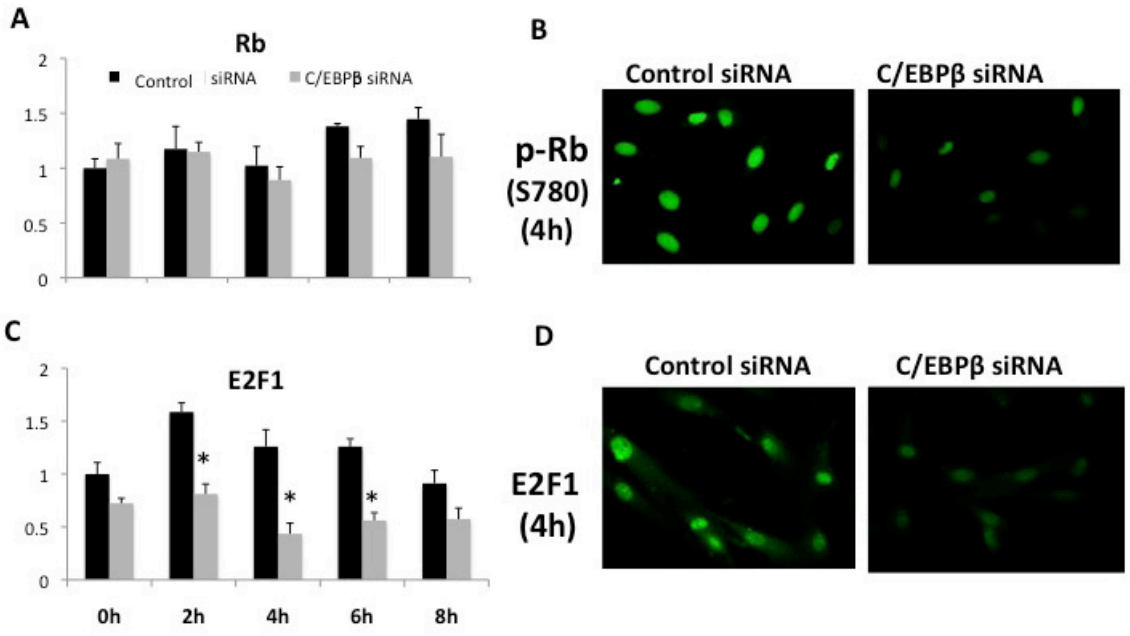

Figure 3.5 Impairment of Rb phosphorylation and E2F1 expression in C/EBPβ-deficient HESCs

HESCs were synchronized at G1 phase by double thymidine block and then transfected with siRNA (40nM) targeted against C/EBPβ or control siRNA (scrambled) for 36 h with thymidine present in the culture. Following transfection, cells were washed and placed in fresh medium containing 5% FBS and then harvested at different times.

Panels A and C: Total RNA was isolated from these cells and the expression of mRNAs corresponding to Rb (A) and E2F1 (C) was monitored by real time PCR, using gene-specific primers. 36B4 was used as an internal control. The relative expression of these genes at different times was determined by normalizing with respect to the level in control siRNA-treated cells at 0 h. The values represent mean ± S.D of three separate measurements. * indicates statistically significant change ($P<0.05$) when compared with control siRNA treatment. Control siRNA-treated: black bar and C/EBPβ siRNA-treated: grey bar.

Panels B and D: The cells were fixed and subjected to immunocytochemistry using antibodies against pRb (S780) (B) and E2F1 (D).

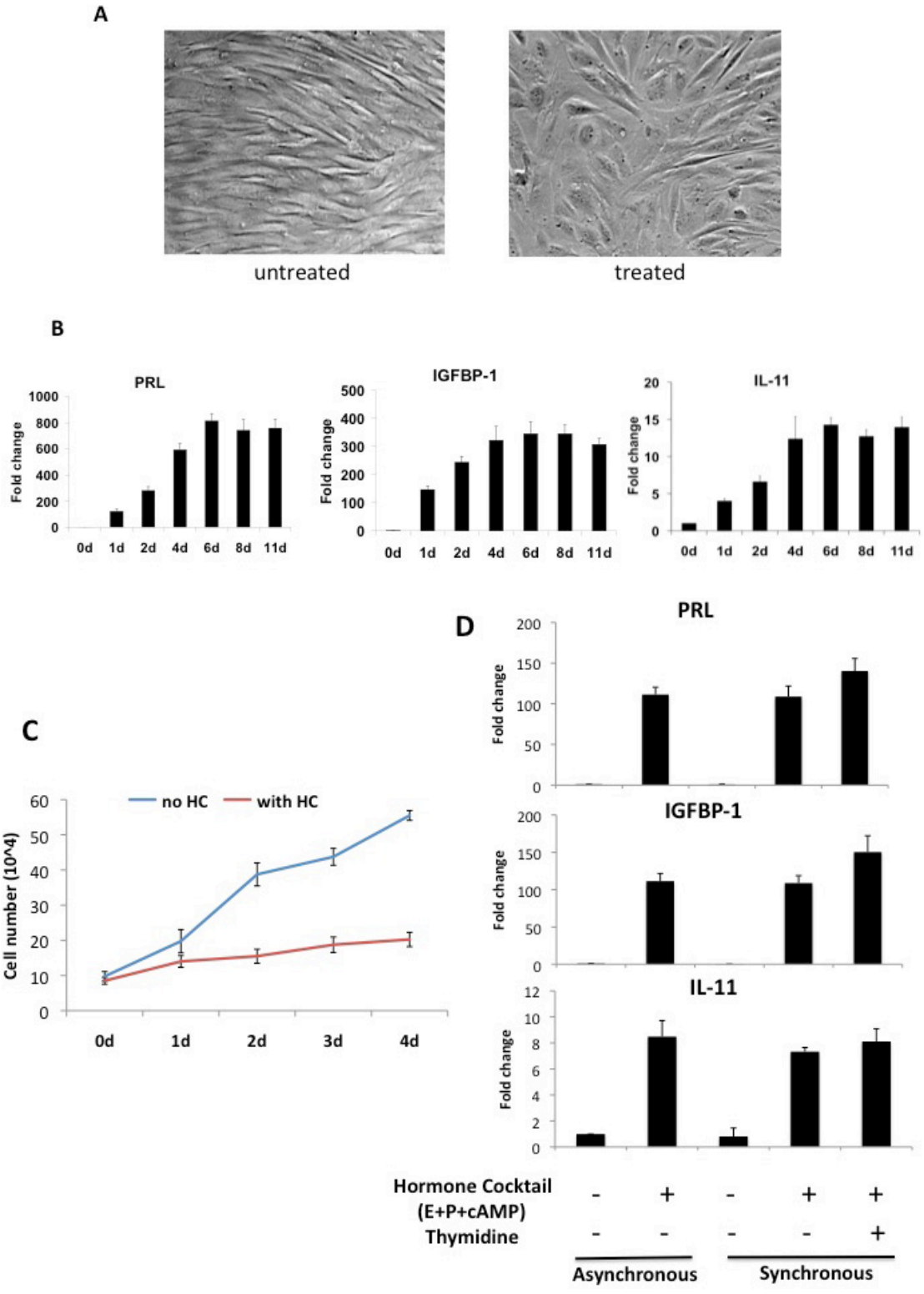

Figure 3.6 In vitro decidualization of HESCs: analysis of proliferation and differentiation

Fig. 3.6 (continued) In vitro decidualization of HESCs: analysis of proliferation and differentiation

Primary cultures of HESCs were established from endometrial biopsies of normal fertile women. The cells were grown in DMEM/F-12 (1:1) medium containing 5% FBS. The cells were treated with or without a hormone cocktail containing 0.5mM 8-bromo-cAMP, 1μM P and 10nM E for 0-11 days.

A. When the cells were examined morphologically after 6 days of culture, a distinct transition from fibroblastic to a plump, epitheloid phenotype, characteristic of decidual cells, was observed.

B. The cells were harvested at different times after addition of hormone cocktail. Total RNA was isolated and subjected to real time PCR using gene-specific primers for PRL, IGFBP-1, and IL-11. "0h" indicates cells immediately prior to hormone cocktail addition. All PCR data were normalized with respect to the mRNA level of 36B4. The relative fold change (mean ± SD) at different time points compared to the 0 h control is shown. No significant alteration in expression of any of the above genes was noted when cultures were maintained in the absence of hormone for 11 days**.**

C. HESCs were cultured in DMEM/F12 containing 5% FBS until they reached 50% confluency. The cells were treated with or without a hormone cocktail containing 0.5mM 8-bromo-cAMP, 1μM P and 10nM E for 0-4 days. At different time points, the cells were harvested by trypsinization and counted using hemocytometer. The cell numbers were determined from three independent plates and mean ± SD are shown. Untreated: blue line, hormone cocktail treated: red line.

D. HESCs were first synchronized via double thymidine block and then treated with the hormone cocktail for 3 days with or without removal of the block. In parallel experiments, asynchronous HESCs were treated with hormone cocktail for 3 days. Total RNA was isolated from cells under different treatment conditions and the expression of decidualization markers, PRL, IGFBP-1 and IL-11 was monitored by real time PCR. All PCR data were normalized with respect to the mRNA level of 36B4. The relative fold change (mean ± SD) at different time points compared to respective untreated control is shown.

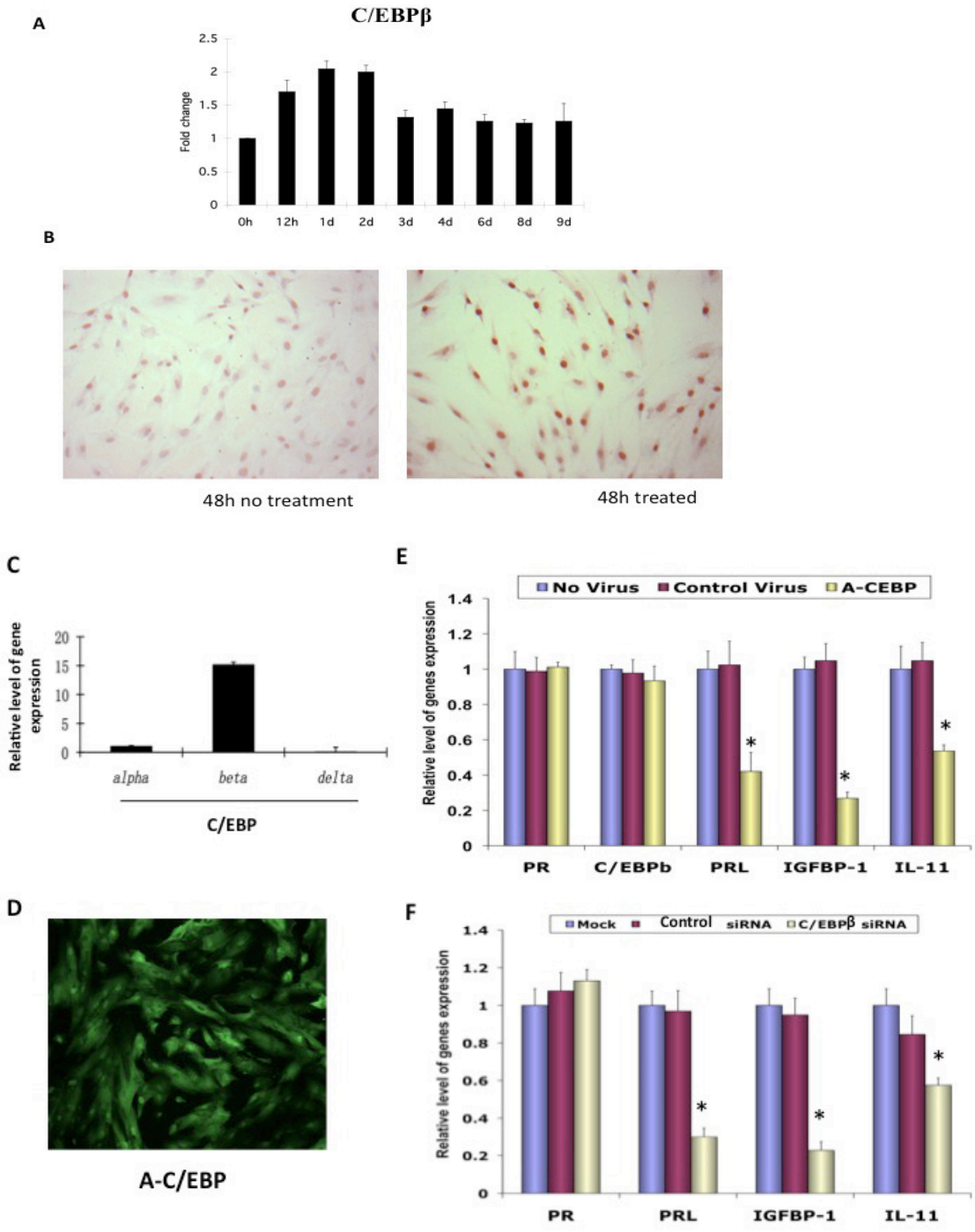

Figure 3.7 C/EBPβ regulates HESC differentiation

HESCs were treated with a hormone cocktail containing 0.5mM 8-bromo-cAMP, 1μM P and 1nM E for 0-9 days.

Fig. 3.7 (continued) C/EBPβ regulates HESC differentiation

A. The cells were harvested at different times after addition of hormone cocktail. Total RNA was isolated and subjected to real time PCR using gene-specific primers for C/EBPβ.

B. Immunocytochemical staining of C/EBPβ in HESCs at 48 h in the presence or absence of hormone cocktail. Nuclear staining of C/EBPβ is observed.

C, HESCs were treated with hormone cocktail for 3 days. RNA was isolated and the expression of C/EBP family members, C/EBPα, C/EBPβ, and C/EBPδ, was analysis by real time PCR using gene-specific primers. Relative expression levels are shown with the level of C/EBPα taken as 1.

D. HESCs (at ~80% confluency) were transduced with adenovirus expressing dominant-negative A-C/EBP at a MOI of 10:1). 24 h after virus addition, the cells were examined by immunocytochemistry using an anti-HA antibody.

E. HESCs were transduced with adenovirus expressing dominant-negative A-C/EBP or control adenovirus. Cells were transduced with adenovirus for 24 h prior to the addition of a hormonal cocktail containing E, P and cAMP. Cells were harvested at 72 h after hormone treatment. Total RNA was isolated and subjected to real time PCR using gene-specific primers for PR, C/EBPβ, PRL, IGFBP-1 and IL-11. Fold-inductions were calculated with respect to the expression levels of these genes in stromal cultures prior to hormone cocktail addition. All real time PCR data were normalized with respect to the mRNA level of 36B4, which encodes a ribosomal protein. * indicates statistically significant change ($P<0.05$) when compared with control (no virus) cells.

F. HESCs were transfected with siRNA (40nM) targeted against C/EBPβ or control (scrambled) siRNA as described in the Materials and Method. 24 h after transfection, cells were treated with hormone cocktail and harvested 72 h after treatment. Total RNA was isolated and subjected to real time PCR using gene-specific primers for PR, PRL, IGFBP-1 and IL-11. Fold-inductions were calculated with respect to the expression levels of these genes in stromal cultures prior to the addition of hormone cocktail. Additionally, all real time PCR data were normalized with respect to the mRNA level of 36B4. *

Fig. 3.7 (continued) C/EBPβ regulates HESC differentiation

indicates statistically significant change ($P<0.05$) when compared with control cells subjected to mock transfection.

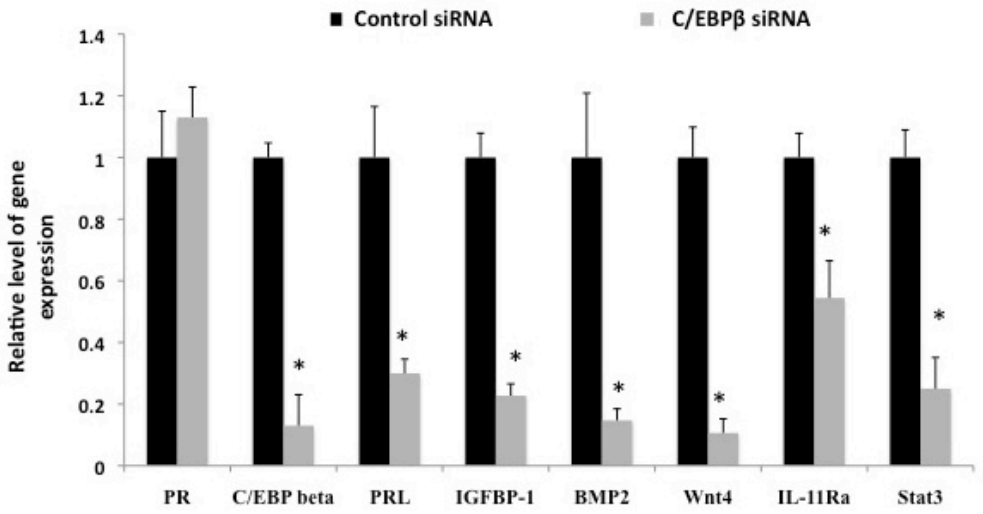

Figure 3.8 C/EBPβ regulates BMP2/Wnt4 and IL-11 signaling pathways during decidualization.

HESCs were transfected with C/EBPβ-specific or control siRNA (40nM) as described above. 24 h after transfection, HESCs were treated with hormone cocktail for 72 h. Total RNA was isolated and the expression of PR, C/EBPβ, PRL, IGFBP-1, BMP2, Wnt4, IL-11Rα and STAT3 was analyzed by real time PCR using gene-specific primers. Relative level of gene expression was calculated with respect to the expression levels of these genes in cells treated with control siRNA. Additionally, all real time PCR data were normalized with respect to the mRNA level of 36B4. * indicates statistically significant change ($P<0.05$) when compared with cells treated with control siRNA.

Pathway analysis for genes Up-regulated in response to C/EBPβ siRNA (>1.5 fold, p-value<0.1)

Pathway	REFLIST (n=25431)	ComLIST (n=967)	ComLIST (expected)	ComLIST (P value)	Genes Involved
Integrin signalling pathway	227	30	8.63	1.48E-06	ITGA1,ITGA2,ITGA4.ITGB3,ITGB5, ILK,COLs,FN1,
Inflammation mediated by chemokine and cytokine signaling pathway	315	30	11.98	1.20E-03	CXCR4, ITGAs, IL8, TNC, RGS4,
Wnt signaling pathway	348	23	13.23	1.00E+00	FZDs, SMARCA4,CDHs,
Cytoskeletal regulation by Rho GTPase	111	23	4.22	2.32E-08	DSTN, TUBBs,
p53 pathway	136	21	5.17	2.05E-05	CDC2. AKT3, GADD45B,ATM, HDAC2, CDKN1A,
FGF signaling pathway	148	18	5.63	3.91E-03	PLCG1, GRB2, MAPK8,
PDGF signaling pathway	189	16	7.19	4.94E-01	AKT3, SRF, ERF, GRB2, PDGFB
Angiogenesis	229	16	8.71	1.00E+00	PLCG1, NOTCH2, TGFB1I1, PDGFB, PDGFC,
EGF receptor signaling pathway	150	15	5.7	1.36E-01	AKT3, PLCG1, ATM,
Apoptosis signaling pathway	131	15	4.98	3.44E-02	ATF3, AKT3, MAPK8,
Ras Pathway	91	13	3.46	1.08E-02	RAC3, AKT3, RHOB,
TGF-beta signaling pathway	154	12	5.86	1.00E+00	TGFBR2, SMAD6, SMAD7, BMPR2, TGFB2,
T cell activation	111	11	4.22	6.85E-01	RAC3, CALM1, AKT3, PLCG1, CALM3,
B cell activation	86	8	3.27	1.00E+00	RAC3, CALM1, CALM3, MAPK8,
Notch signaling pathway	51	5	1.94	1.00E+00	NOTCH2, ADAM17,
VEGF signaling pathway	80	4	3.04	1.00E+00	AKT3, PLCG1, TGFB1I1

Table 3.1 Signaling pathways down regulated in HESCs in response to C/EBPβ siRNA

The microarray data, showing molecular pathways down regulated in response to C/EBPβ-specific siRNA, were analyzed by Panther Classification System. REFLIST indicates the number of genes in NCBI H. sapiens database that are known to be involved in a particular pathway. ComLIST indicates the actual number of genes that are altered in this pathway upon siRNA treatment. ComLIST (expected) is the number of genes that are predicted to be affected by siRNA treatment. ComLIST (p-value) indicates the statistical analysis of the number of genes actually altered in the particular pathway.

Pathway analysis for genes down-regulated in response to C/EBPβ siRNA (>1.5 fold, p-value<0.1)

Pathway	REFLIST (n=25431)	ComLIST (n=923)	ComLIST (expected)	ComLIST (P value)	Gene Involved
Inflammation mediated by chemokine and cytokine signaling pathway	315	22	11.43	5.51E-01	IL11Ra, STAT3, SOCS3, SOCS5, IFNGR1, CCL2, ITPRs, CCL8, PTGS1, PTGS2, IL15
Angiogenesis	229	17	8.31	8.55E-01	FGFR1, FZD1, STAT3, VEGFA, PDGFRA, PDGFD, SPHK1,
PDGF signaling pathway	189	16	6.86	3.14E-01	STAT3, ITPRs, STAT5A, STAT5B, SHC3,
WNT signaling pathway	348	15	12.63	1.00E+00	FAT4, FZD1, HOXA5, ITPRs, WNT4, WNT2, NFATC4, SMARCD3
Interleukin signaling pathway	194	15	7.04	9.66E-01	FOXD1, IL15, IL15R, IL11RA, STAT3, IL6R, IL13RA, STAT5A, STAT5B, IL6ST
Integrin signalling pathway	227	14	8.24	1.00E+00	COLs, LAMB2, LAMC2, LAMC1, MAPKs,
EGF receptor signaling pathway	150	14	5.44	2.46E-01	AREG, EREG, STAT3, MAPKs, SHC, STAT5A, STAT5B
Apoptosis signaling pathway	131	14	4.75	6.85E-02	FAS, ATF1, NFKB1, MAPKs,
T cell activation	111	9	4.03	1.00E+00	NCK1, ITPR3, MAPKs, NFATC4,
Endothelin signaling pathway	98	9	3.56	1.00E+00	ECE1, ITPRs, ADCY7, PTGS2,
B cell activation	86	9	3.12	7.98E-01	NFKBIA, ITPRs, MAPKs, NFATC4,
Oxidative stress response	69	8	2.5	7.02E-01	MKNK2, MAPKs, STAT1,
Interferon-gamma signaling pathway	35	8	1.27	8.91E-03	IFNGR1, SOCSs, STAT1, MAPKs,
FGF signaling pathway	148	8	5.37	1.00E+00	FGFR1, MAPKs, SHC3,
TGF-beta signaling pathway	154	7	5.59	1.00E+00	BMP2, FOXD1,FOXL2, MAPKs, SKI,
JAK/STAT signaling pathway	23	7	0.83	4.42E-03	STAT3, STAT1, STAT5A, STAT5B, MAPK14,
VEGF signaling pathway	80	5	2.9	1.00E+00	VEGFA, PRKCH, SPHK1, MAPK14,

Table 3.2 Signaling pathways up regulated in HESCs in response to C/EBPβ siRNA

The microarray data, showing molecular pathways up regulated in response to C/EBPβ-specific siRNA, were analyzed by Panther Classification System. REFLIST indicates the number of genes in NCBI H. sapiens database that are known to be involved in a particular pathway. ComLIST indicates the actual number of genes that are altered in this pathway upon siRNA treatment. ComLIST (expected) is the number of genes that are predicted to be affected by siRNA treatment. ComLIST (p-value) indicates the statistical analysis of the number of genes actually altered in the particular pathway.

References

1. **Giudice LC, Telles TL, Lobo S, Kao L** 2002 The molecular basis for implantation failure in endometriosis: On the road to discovery. Ann NY Acad Sci 955:252-264
2. **Bell SC** 1990 Decidualization and relevance to menstruation. Cambridge, U. K.: Cambridge University Press
3. **Navot D, Scott RT, Droesch K** 1991 The window of embryo transfer and the efficiency of human conception in vitro. Fertil Steril 55:114-118
4. **Maruyama T, Yoshimura Y** 2008 Molecular and cellular mechanisms for differentiation and regeneration of the uterine endometrium. Endocrine journal
5. **Ryan IP, Taylor RN** 1997 Endometriosis and infertility: new concepts. Obstet Gynecol Surv 52:365-371
6. **Telgmann R, Maronde E, Tasken K, Gellersen B** 1997 Activated protein kinase A is required for differentiation-dependent transcription of the decidual prolactin gene in human endometrial stromal cells. Endocrinology 138:929-937
7. **Dimitriadis E, Stoikos C, Baca M, Fairlie WD, McCoubrie JE, Salamonsen LA** 2005 Relaxin and prostaglandin E(2) regulate interleukin 11 during human endometrial stromal cell decidualization. J Clin Endocrinol Metab 90:3458-3465
8. **Kim JJ, Buzzio OL, Li S, Lu Z** 2005 Role of FOXO1A in the regulation of insulin-like growth factor-binding protein-1 in human endometrial cells: interaction with progesterone receptor. Biology of reproduction 73:833-839
9. **Li Q, Kannan A, Wang W, Demayo FJ, Taylor RN, Bagchi MK, Bagchi IC** 2007 Bone morphogenetic protein 2 functions via a conserved signaling pathway involving Wnt4 to regulate uterine decidualization in the mouse and the human. The Journal of biological chemistry 282:31725-31732
10. **Takano M, Lu Z, Goto T, Fusi L, Higham J, Francis J, Withey A, Hardt J, Cloke B, Stavropoulou AV, Ishihara O, Lam EW, Unterman TG, Brosens JJ, Kim JJ** 2007 Transcriptional cross talk between the forkhead transcription factor forkhead box O1A and the progesterone receptor coordinates cell cycle regulation and differentiation in human endometrial stromal cells. Molecular endocrinology (Baltimore, Md 21:2334-2349
11. **Mantena SR, Kannan A, Cheon YP, Li Q, Johnson PF, Bagchi IC, Bagchi MK** 2006 C/EBPbeta is a critical mediator of steroid hormone-regulated cell proliferation and differentiation in the uterine epithelium and stroma. Proceedings of the National Academy of Sciences of the United States of America 103:1870-1875
12. **Ramji DP, Foka P** 2002 CCAAT/enhancer-binding proteins: structure, function and regulation. Biochem J 365:561-575
13. **Sterneck E, Tessarollo L, Johnson PF** 1997 An essential role for C/EBPbeta in female reproduction. Genes & development 11:2153-2162

14. **Pall M, Hellberg P, Brannstrom M, Mikuni M, Peterson CM, Sundfeldt K, Norden B, Hedin L, Enerback S** 1997 The transcription factor C/EBP-beta and its role in ovarian function; evidence for direct involvement in the ovulatory process. EMBO J 16:5273-5279

15. **Nalbant D, Williams SC, Stocco DM, Khan SA** 1998 Luteinizing hormone-dependent gene regulation in Leydig cells may be mediated by CCAAT/enhancer-binding protein-beta. Endocrinology 139:272-279

16. **Greenbaum LE, Li W, Cressman DE, Peng Y, Ciliberto G, Poli V, Taub R** 1998 CCAAT enhancer- binding protein beta is required for normal hepatocyte proliferation in mice after partial hepatectomy. J Clin Invest 102:996-1007

17. **Tanaka T, Yoshida N, Kishimoto T, Akira S** 1997 Defective adipocyte differentiation in mice lacking the C/EBPβ and /or C/EBPδ gene. EMBO J 16:7432-7443

18. **Tang QQ, Otto TC, Lane MD** 2003 CCAAT/enhancer-binding protein beta is required for mitotic clonal expansion during adipogenesis. Proceedings of the National Academy of Sciences of the United States of America 100:850-855

19. **Robinson GW, Johnson PF, Hennighausen L, Sterneck E** 1998 The C/EBPbeta transcription factor regulates epithelial cell proliferation and differentiation in the mammary gland. Genes & development 12:1907-1916

20. **Seagroves TN, Krnacik S, Raught B, Gay J, Burgess-Beusse B, Darlington GJ, Rosen JM** 1998 C/EBPbeta, but not C/EBPalpha, is essential for ductal morphogenesis, lobuloalveolar proliferation, and functional differentiation in the mouse mammary gland. Genes & development 12:1917-1928

21. **Tanaka T, Akira S, Yoshida K, Umemoto M, Yoneda Y, Shirafuji N, Fujiwara H, Suematsu S, Yoshida N, Kishimoto T** 1995 Targeted disruption of the NF-IL6 gene discloses its essential role in bacteria killing and tumor cytotoxicity by macrophages. Cell 80:353-361

22. **Zhu S, Yoon K, Sterneck E, Johnson PF, Smart RC** 2002 CCAAT/enhancer binding protein-beta is a mediator of keratinocyte survival and skin tumorigenesis involving oncogenic Ras signaling. Proceedings of the National Academy of Sciences of the United States of America 99:207-212

23. **Harper JV** 2005 Synchronization of cell populations in G1/S and G2/M phases of the cell cycle. Methods Mol Biol 296:157-166

24. **Hendzel M, Wei Y, Mancini MA, Hooser AV, Ranalli T, Brinkley BR, Bazett-Jones, Allis CD** 1997 Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. Chromosoma 106:348-360

25. **Gu Y, Rosenblatt J, Morgan DO** 1992 Cell cycle regulation of CDK2 activity by phosphorylation of Thr160 and Tyr15. EMBO J 11:3995-4005

26. **Dyson N** 1998 The regulation of E2F by pRB-family proteins. Genes Dev 12:2245-2262

27. **Lundberg AS, Weinberg RA** 1998 Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. Mol Cell Biol 18:753-761

28. **Ezhevsky SA, Ho A, Becker-Hapak M, Davis PK, Dowdy SF** 2001 Differential regulation of retinoblastoma tumor suppressor protein by G(1) cyclin-dependent kinase complexes in vivo. Mol Cell Biol 21:4773-4784

29. **Naderi S, Wang JY, Chen TT, Gutzkow KB, Blomhoff HK** 2005 cAMP-mediated inhibition of DNA replication and S phase progression: involvement of Rb, p21Cip1, and PCNA. Mol Biol Cell 16:1527-1542

30. **Huang JR, Tseng L, Bischof P, Janne OA** 1987 Regulation of prolactin production by progestin, estrogen, and relaxin in human endometrial stromal cells. Endocrinology 121:2011-2017

31. **Bell SC, Jackson JA, Ashmore J, Zhu HH, Tseng L** 1991 Regulation of insulin-like growth factor-binding protein-1 synthesis and secretion by progestin and relaxin in long term cultures of human endometrial stoma cells. J Clin Endocrinol Metab 72:1014-1024

32. **Giudice LC, Milkowski DA, Lamson G, Rosenfeld RG, Irwin JC** 1991 Insulin-like growth factor-binding proteins in human endometrium: steroid-dependent messenger ribonucleic acid expression and protein synthesis. J Clin Endocrinol Metab 72:779-787

33. **Christian M, Mak I, White JO, Brosens JJ** 2002 Mechanisms of decidualization. Reprod Biomed Online 4 Suppl 3:24-30

34. **Greenwel P, Tanaka S, Penkov D, Zhang W, Olive M, Moll J, Vinson C, Di Liberto M, Ramirez F** 2000 Tumor necrosis factor alpha inhibits type I collagen synthesis through repressive CCAAT/enhancer-binding proteins. Mol Cell Biol 20:912-918

35. **Zhang JW, Tang QQ, Vinson C, Lane MD** 2004 Dominant-negative C/EBP disrupts mitotic clonal expansion and differentiation of 3T3-L1 preadipocytes. Proceedings of the National Academy of Sciences of the United States of America 101:43-47

36. **Vinson C, Myakishev M, Acharya A, Mir AA, Moll JR, Bonovich M** 2002 Classification of human B-ZIP proteins based on dimerization properties. Mol Cell Biol 22:6321-6335

37. **Pohnke Y, Kempf R, Gellersen B** 1999 CCAAT/enhancer-binding proteins are mediators in the protein kinase A-dependent activation of the decidual prolactin promoter. The Journal of biological chemistry 274:24808-24818

38. **Lee KY, Jeong JW, Wang J, Ma L, Martin JF, Tsai SY, Lydon JP, DeMayo FJ** 2007 Bmp2 is critical for the murine uterine decidual response. Mol Cell Biol 27:5468-5478

39. **Robb L, Li R, Hartley L, Nandurkar HH, Koentgen F, Begley CG** 1998 Infertility in female mice lacking the receptor for interleukin 11 is due to a defective uterine response to implantation. Nature medicine 4:303-308

40. **Bilinski P, Roopenian D, Gossler A** 1998 Maternal IL-11Ralpha function is required for normal decidua and fetoplacental development in mice. Genes & development 12:2234-2243

41. **Nakamura H, Kimura T, Koyama S, Ogita K, Tsutsui T, Shimoya K, Taniguchi T, Koyama M, Kaneda Y, Murata Y** 2006 Mouse model of human infertility: transient and local inhibition of endometrial STAT-3 activation results in implantation failure. FEBS letters 580:2717-2722

42. **Catalano RD, Johnson MH, Campbell EA, Charnock-Jones DS, Smith SK, Sharkey AM** 2005 Inhibition of Stat3 activation in the endometrium prevents implantation: a nonsteroidal approach to contraception. Proceedings of the National Academy of Sciences of the United States of America 102:8585-8590

43. **Gellersen B, Brosens IA, Brosens JJ** 2007 Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives. Semin Reprod Med 25:445-453

44. **Gellersen B, Brosens J** 2003 Cyclic AMP and progesterone receptor cross-talk in human endometrium: a decidualizing affair. J Endocrinol 178:357-372

45. **Plante BJ, Kannan A, Bagchi MK, Yuan L, Young SL** 2009 Cyclic regulation of transcription factor C/EBP beta in human endometrium. Reprod Biol Endocrinol 7:15

46. **Tang B, Guller S, Gurpide E** 1993 Cyclic adenosine 3',5'-monophosphate induces prolactin expression in stromal cells isolated from human proliferative endometrium. Endocrinology 133:2197-2203

47. **Irwin JC, Kirk D, King RJB, Quigly MM, Gwatkin RBL** 1989 Hormonal regulation of human endometrial stromal cells in culture: an in vitro model for decidualization. Fertil Steril 52:761-768

48. **Ryan IP, Schriock ED, Taylor RN** 1994 Isolation, characterization, and comparison of human endometrial and endometriosis cells in vitro. J Clin Endocrinol Metab 78:642-649

49. **Harper JV, Brooks G** 2004 The mammalian cell cycle. Methods in Molecular Biology 296:113-153

50. **Nurse P** 2000 A long twentieth century of the cell cycle and beyond. Cell 100:71-78

51. **Alberts B** 2002 Molecular Biology of the Cell. New York: Science

52. **Brooks G** 2004 Cyclins, cyclin-dependent kinases, and cylcin-dependent kinase inhibitors Methods in Molecular Biology 296:291-298

53. **Sherr CJ, Roberts JM** 2004 Living with or without cyclins and cyclin-dependent kinases. Genes & development 18:2699-2711

54. **Zhu W, Giangrande PH, Nevins JR** 2004 E2Fs link the control of G1/S and G2/M transcription. EMBO J 23:4615-4626

55. **Christian M, Zhang X, Schneider-Merck T, Unterman TG, Gellersen B, White JO, Brosens JJ** 2002 Cyclic AMP-induced forkhead transcription factor, FKHR, cooperates with CCAAT/enhancer-binding protein beta in differentiating human endometrial stromal cells. The Journal of biological chemistry 277:20825-20832

56. **Poli V** 1998 The role of C/EBP isoforms in the control of inflammatory and native immunity functions. The Journal of biological chemistry 273:29279-29282

57. **Heinrich PC, Behrmann I, Muller-Newen G, Schaper F, Graeve L** 1998 Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. Biochem J 334 (Pt 2):297-314

58. **Dimitriadis E, Salamonsen LA, Robb L** 2000 Expression of interleukin-11 during the human menstrual cycle: coincidence with stromal cell decidualization and relationship to leukaemia inhibitory factor and prolactin. Molecular human reproduction 6:907-914

59. **Dimitriadis E, Robb L, Salamonsen LA** 2002 Interleukin 11 advances progesterone-induced decidualization of human endometrial stromal cells. Molecular human reproduction 8:636-643

60. **Cork BA, Li TC, Warren MA, Laird SM** 2001 Interleukin-11 (IL-11) in human endometrium: expression throughout the menstrual cycle and the effects of cytokines on endometrial IL-11 production in vitro. J Reprod Immunol 50:3-17

61. **Dimitriadis E, Sharkey AM, Tan YL, Salamonsen LA, Sherwin JR** 2007 Immunolocalisation of phosphorylated STAT3, interleukin 11 and leukaemia inhibitory factor in endometrium of women with unexplained infertility during the implantation window. Reprod Biol Endocrinol 5:44

62. **Dimitriadis E, Stoikos C, Stafford-Bell M, Clark I, Paiva P, Kovacs G, Salamonsen LA** 2006 Interleukin-11, IL-11 receptoralpha and leukemia inhibitory factor are dysregulated in endometrium of infertile women with endometriosis during the implantation window. J Reprod Immunol 69:53-64

63. **Karpovich N, Klemmt P, Hwang JH, McVeigh JE, Heath JK, Barlow DH, Mardon HJ** 2005 The production of interleukin-11 and decidualization are compromised in endometrial stromal cells derived from patients with infertility. J Clin Endocrinol Metab 90:1607-1612

CHAPTER 4

Signal Transducer and Activator of Transcription 3 (STAT3) is a Target of C/EBPβ and Controls Human Endometrial Stromal Decidualization

4.1 Abstract

Our earlier studies revealed that the transcription factor C/EBPβ plays an essential role in human endometrial stromal cell (HESC) differentiation. In the present study, we undertook the identification of the C/EBPβ-regulated gene networks that control HESC differentiation in an *in vitro* decidualization system. We attenuated C/EBPβ expression by siRNA strategy and then performed gene expression profiling, using normal and C/EBPβ-deficient HESCs. Our studies revealed that signal transducer and activator of transcription 3 (STAT3), which mediates signaling by several cytokines including IL-11, is a prominent downstream target of C/EBPβ. Chromatin immunoprecipitation experiments indicated that C/EBPβ controls the expression of STAT3 by directly interacting with its promoter. siRNA-mediated down regulation of STAT3 expression in HESCs resulted in significantly reduced differentiation of these cells, indicating an important role for this cytokine signal transducer in the decidualization process. Next, we performed gene expression analysis to identify specific genes under STAT3 control during HESC differentiation. We found that the expression of the enzyme spingosine kinase 1 (SphK1), an important angiogenic and inflammatory factor, is strongly inhibited in the absence of STAT3. siRNA-mediated suppression of SphK1 mRNA levels led to a block in the HESC differentiation process. This blockade could be reversed upon the addiction of spingosine 1 phosphate, the product of SphK1. Collectively, our findings provided a novel link between C/EBPβ- and STAT3-dependent cytokine signaling pathways that regulate endometrial functions during decidualization. Our study uncovered a unique linear pathway involving C/EBPβ, STAT3 and the lipid metabolite spingosine 1 phosphate, and indicated that it serves as a regulator of critical biological processes during decidualization.

4.2 Introduction

The human endometrium undergoes extensive remodeling during the menstrual cycle, which is strictly controlled by the ovarian steroid hormones, estrogen (E) and progesterone (P), acting via their cognate receptors. The postovulatory surge of progesterone induces sequential morphological and biochemical changes in the stromal compartment, which are critical for embryo implantation, and the growth and development of fetus during early pregnancy. In the human, this transformation, known as pre-decidualization, is initiated around the spiral arteries at mid-late secretory phase (cycle day 20-24) and involves the conversion of fibroblast-like stromal cells into large, rounded and secretory decidual cell. Following embryo implantation, decidualization progresses further, leading to the formation of the pregnancy decidua (1;2). The decidualizing human endometrial stromal cells (HESCs) produce numerous biological molecules, including growth factors, cytokines, chemokines, and extracellular matrix components, to regulate trophoblast invasion and to attenuate the maternal immune response to the fetus (3-6). Impaired decidual progression causes infertility, recurrent miscarriages, and disease states such as endometriosis and endometrial cancer (3;7-10).

To date, development of transgenic mouse models helped to establish functional roles of several genes in the decidualization process. These include the progesterone recptor A(11), homeobox genes HoxA10 and Hoxa11 (12;13), cycloxygenase 2(14), interleukin 11 receptor alpha(15), CCAAT/enhancer binding protein beta(16), Indian hedgehog(17), steroid receptor coactivator 2(18) etc. Studies employing an in vitro system in which HESCs isolated from the proliferative phase of the cycle undergo differentiation in the presence of a hormonal cocktail containing E, P and cAMP analog confirmed that several genes, such as PR (19) and forehead box O1 (FOXO1)(20), exert important functions during the decidualization process in the human.

Our previous studies have shown that C/EBPβ plays a critical role in human endometrial stromal decidualization. We performed gene expression profiling using DNA microarrays to identify the downstream pathways regulated by this transcription factor during stromal differentiation. Our studies revealed that several key signaling molecules, such as BMP2, Wnt4 and VEGFA, operate downstream of C/EBPβ during

decidualization. Additionally, the expression of the IL-11 receptor α and its signal transducer STAT3 was also markedly reduced upon the attenuation of C/EBPβ expression. It is of interest that female mice bearing a null mutation in the gene encoding the IL-11 receptor α are infertile due to defective decidualization and improper trophoblast invasion (15;21). In the human, IL-11 is maximally expressed during decidualization, while its receptor is also present in the decidualizing stromal cell (22). It was reported that aberrant production of IL-11 by the endometrium, resulting in compromised decidualization, is associated with certain types of infertility (3;9).

STAT3 is an important signaling mediator for several cytokines, such as IL-6, IL-11, and leukemia inhibitory factor, which play important roles in immune modulation, angiogenesis, and uterine receptivity (23-26). Binding of the cytokines to their specific cell surface receptors attracts and activates Janus tyrosine kinases (JAKs), which phosphorylate the cytokine receptors, thereby creating active docking sites for STATs. Activated STAT3 plays critical roles in angiogenesis and immuno suppression by regulating downstream target genes, such as VEGFA (23;27-29). Earlier studies in the mouse indicated that transient blockade of STAT3 expression or function prior to implantation significantly reduced the embryo implantation (30;31). *In vivo* immuno-localization studies have shown that STAT3 is induced and activated in stromal compartment during implantation (26;32;33). Collectively, these results are consistent with a potential role of the STAT3 signaling pathway downstream of C/EBPβ during human stromal decidualization.

In this study, we investigated the mechanisms by which STAT3 mediates the function of C/EBPβ during HESC decidualization. We observed that the enhanced expression of C/EBPβ protein during human endometrial stromal decidualization immediately precedes increased activation of STAT3 signaling. Employing a siRNA strategy, we confirmed that C/EBPβ regulates STAT3 mRNA and protein expression. Our studies using ChIP further revealed that C/EBPβ directly interacts with the STAT3 promoter to regulate its activity. A loss of function approach using siRNAs demonstrated that STAT3 is critical for differentiation of HESCs. Using gene expression profiling, we identified STAT3-regulated downstream pathway during stromal differentiation. Our

studies showed that SphK1, a key angiogenic and immuno- modulatory molecule, operates downstream of STAT3 to control decidualization. Collectively, these studies uncovered a unique linear signaling pathway involving C/EBPβ, STAT3, and SphK1, which controls decidualization of HESCs and is a likely regulator of angiogenesis and immune modulation during early pregnancy.

4.3 Materials and Methods

Primary human endometrial stromal culture and in vitro decidualization

The human endometrial stromal cells (HESCs) were provided by Dr. Robert Taylor of Emory University Medical School. These cells were isolated from biopsies collected from the proliferative stage endometrium of normal cycling women and cultured in DMEM/F-12 medium (Invitrogen) containing 5% (v/v) fetal bovine serum (Hyclone), 50 μg/ml penicillin, and 50 μg/ml streptomycin (Invitrogen). To induce decidualization, the cells were treated with a hormone cocktail, containing 0.5 mM 8-bromo-adenosine-3', 5'-cyclic monophosphate (8-Br-cAMP) (Sigma), 1 μM progesterone (Sigma) and 10 nM 17ß-estradiol (Sigma). The medium was changed every 48 h and the culture was maintained up to 12 days.

Immunocytochemistry

HESCs were fixed in formalin solution (Sigma) at room temperature for 10 min followed by washing with PBS for 10 min. The cells were permeabilized by 0.25% Triton X-100 in PBS for 10 min and non-specific binding of antibodies was blocked with 10% donkey serum for 1 h at room temperature. Primary antibodies included anti- C/EBPβ (C-19, Santa Cruz Biotech), anti-STAT3 (C-20, Santa Cruz Biotech), and anti-phospho-STAT3 (Ser-727, Cell Signaling Corp.). Incubations with these antibodies were performed overnight at 4°C. Fluorescence (Cy3)-conjugated anti-mouse IgG or anti-rabbit IgG (Jackson Lab) was used as secondary antibody. DAPI (1ug/ml in PBS) was used as counter staining

Adenovirus-mediated expression of dominant negative C/EBPβ

An adenoviral vector expressing a dominant negative mutant of C/EBPβ (A-C/EBP) was provided by Dr. Charles Vinson (NCI/NIH). This vector expresses A-C/EBP under the control of CMV promoter. The protein is expressed with a HA epitope tag. An adenoviral vector lacking the A-C/EBP insert and expressing GFP was used as a control. Control or A-C/EBP-carrying adenovirus was added to HESC monolayer (MOI: 10:1) at 80%-90% confluency. After 24 h of viral transduction, the viral particles were removed and the cells were treated with a hormone cocktail to induce decidualization.

siRNA transfection

The HESCs were transfected with siRNA against C/EBPβ, STAT3 or scrambled siRNA as control (Silencer select pre-designed, Ambion), following the manufacturer's protocol (Silentfect, Bio-Rad Laboratories). Briefly, SilentFect transfection reagent was mixed with 40 nM of siRNA and added to HESCs at 80% confluency. After 24 h, siRNA was removed and cells were treated with a hormone cocktail to induce decidualization. Cells were harvested after 3 days of hormone cocktail treatment. Gene expression was examined by quantitative real-time PCR using gene-specific primers.

Quantitative real-time PCR analysis

Total RNA was extracted from cultured HESCs using the TRIzol RNA purification kit (Invitrogen), according the manufactures instructions. Reverse transcription was performed using the SuperScript Fist-Strand Synthesis System for RT-PCR (Invitrogen), following the manufactures instructions. The expression of mRNAs corresponding to PR, C/EBPβ, IGFBP-1, PRL, STAT3, IL-11, IL-11Rα, SphK1 and SphK2 was examined by quantitative real-time PCR analysis using SYBR-Green supermix (Bio-Rad Laboratories) and gene-specific primers. The expression level of a gene at a given time point or under a certain treatment condition was quantified as a fold change (mean±SD) relative to its level at 0 h or untreated condition after normalization with respect to the internal control 36B4.

The primer sets used in real-time PCR experiments are listed below:

Gene	Sense Primer	Anti-sense Primer
PR	TGCCTATCCTGCCTCTCAATCAC	CGCCGTCGTAACTTTCGTCTTC
C/EBPβ	AACTCTCTGCTTCTCCCTCTG	TGCGTCAGTCCCGTGTAC
PRL	CTACATCCATAACCTCTCCTCAG	GGGCTTGCTCCTTGTCTTC
IGFBP-1	TTATCACAGCAGACAGGTAGG	TGGCTTGGCAGGAATGTAG
IL-11	GCTGCACCTGACAGTTGACT	CGCCCCCAGTACTGAAATAA
IL-11Rα	AAGCAGCCGACTATGAGAAC	TGTAGGAGGTGAGGTAGCG
STAT3	ACAGGTGAGGCAGAACAG	CCATACGCACAGGAGAGG
SphK1	CGCTGTGCCTTAGTGTCTAC	AGCATAGCCTCCACCTTCTC
SphK2	ACCTGACTCCTTGCTCCTAC	GGTCCTGCTGCTCCTCTG

PRL assay

The HESCs were cultured in 6-well plates and decidualization was induced as described previously. The culture medium was collected every 48 h and replaced with fresh medium. The PRL production was analyzed by an ELISA kit (Cal-Biotech). The assay was performed in triplicates, according the manufactures instructions. A quality control sample (unused culture medium) was included in each assay. The lower detection limit of the assay was 0.3ng/ml of PRL. The inter-assay and intra-assay variability were 5.8 and 2.3%, respectively.

Chromatin immunoprecipitation (ChIP) analysis.

The ChIP analysis was performed using the EZ ChIP (Upstate Biotechnology), according to the manufacturer's protocol. Briefly, HESCs were treated with hormone cocktail for 3 days to induce the decidualization. Thereafter, cells ($8X10^6$) were placed in PBS buffer and cross-linked with 1% formaldehyde for 10 min. The cross-linked cells were harvested, lysed using SDS lysis buffer, and sonicated. After pre-clearing the lysates with salmon sperm DNA–protein A at 4°C for 2 h, the DNA–protein complexes in the supernatant were immunoprecipitated using antibodies against RNA polymerase II,

Rabbit IgG (Upstate Biotechnology) or C/EBPβ (c-19, Santa Cruz Biotechnology). The immune complexes were recovered by adding protein A–agarose. The beads were then washed repeatedly and the bound complexes were eluted using the elution buffer. The cross-linking was reversed and then proteins were digested using 0.5mg/ml proteinase K. Purified DNA were used as templates for PCR using various primer sets to amplify specific regions of the STAT3 promoter.

For the experiments using the dominant negative mutant A-C/EBP, HESCs were first transduced with control or A/C/EBP adenovirus as described above. After 24 h of transduction, the viral particles were removed and the cells were treated with a hormone cocktail to induce decidualization. Following 3 days of hormonal cocktail treatment, cells were harvested to perform the ChIP assay describe as above.

Microarray Analysis

HESCs isolated from proliferative phase endometrial biopsies were transfected with scrambled siRNA (control) or STAT3-specific siRNA as described above. 24 h after siRNA administration, a hormone cocktail was added to the cells to induce decidualization. The decidualization process was allowed to proceed for 3 days following the addition of the cocktail. Cells were harvested and total RNA was prepared by TRIzol ® Reagent (Invitrogen, CA). RNA samples were processed at the Biotechnology Center of the University of Illinois, Urbana and Champaign. RNA integrity was verified using Agilent 2100 bioanalyser (Agilent Technologies Inc., Santa Clara, CA, USA). Each RNA sample was processed for microarray hybridization using Genechip® Human Geneme U133 A Plus 2.0 arrays (Affymetrix Inc., Santa Clara, CA), following the established protocol. The resulting data files were analyzed by Affymetrix GeneChip® Command Console® (AGCC) software.

Statistical analysis

The RNA and protein samples were prepared from at least three separate primary cultures subjected to the same experimental treatment. The real-time PCR results are expressed as mean ± S.D of three separate measurements. Statistical significance of the

data was determined using one-way ANOVA followed by student t-test. A p-value of <0.05 was considered significant.

4.4 Results

Activation of IL-11 signaling pathway during human endometrial stromal decidualization

Our microarray experiments, described in Chapter III, revealed that IL-11 receptor α is a downstream target of regulation by C/EBPβ in HESCs. This finding raised the possibility that this transcription factor controls cytokine signaling pathways that are critical for decidualization. Previous studies showed that IL-11 is secreted during cAMP-induced decidualization of HESCs and addition of recombinant IL-11 to these cells enhances their P-induced decidualization *in vitro* (34). We analyzed the expression pattern of IL-11, its receptor IL11Rα, and its signaling transducer STAT3 during human stromal decidualization (Fig. 4.1A). Whereas the level of IL-11 mRNA is robustly increased during stromal decidualization and reaches a peak by day 5, the level of IL11Rα was elevated early in the decidualization program and maintained at a high level up to 6 days of culture. The level of STAT3 was enhanced early and reaches a peak by day 3 and day 4 and then gradually declines close to basal level.

In many tissues, cytokines, such as IL11, act through their cell surface receptors to activate the signal transducer STAT3 by phosphorylating it initially at Tyr 705, which allows it to dimerize and enter the nucleus. This is followed by a phosphorylation at Ser 727, which activates its transcriptional function and allows it to modulate downstream gene expression events (28). We, therefore, examined the temporal pattern of generation of activated STAT3 during the *in vitro* decidualization program by using an antibody that specifically recognizes the activating phosphorylation at Ser 727. As shown in Fig. 4.1B, coincident with increasing IL-11 production in differentiating stromal cells, phosphorylated STAT3 is formed and translocated to the nucleus. The phosphorylated state of STAT3 is maintained for several days, although its intensity diminished slightly by day 6. These results are consistent with the hypothesis that IL11 signaling pathway

may play a functional role in decidualization and its downstream actions might be transduced via activation of STAT3.

STAT3 is a downstream target of C/EBPβ during decidualization

As indicated by our microarray analysis described in Chapter III, STAT3 is one of the genes, which are significantly repressed upon suppression of C/EBPβ expression in HESCs in response to treatment with C/EBPβ-specific siRNA. To confirm the results of our microarray analysis, we carried out quantitative real-time PCR analysis, using RNA isolated from the HESCs treated with C/EBPβ-specific or control (scrambled) siRNA. We observed that treatment with C/EBPβ siRNA, which efficiently down regulated the expression of this transcription factor, significantly inhibited the expression of STAT3 as well as that of the classical decidualization markers PRL and IGFBP1 (Fig. 4.2A). Consistent with the observation that STAT3 mRNA is down regulated in C/EBPβ-deficient cells, immunocytochemical analyses showed that the cells transfected with C/EBPβ siRNA exhibited a drastically decreased STAT3 protein expression, compared with the cells transfected with control siRNA (Fig. 4.2B).

To further confirm this regulatory event, we undertook another loss-of-function approach, using a dominant negative mutant of C/EBPβ (A-C/EBP). In this mutant, the basic region critical for DNA binding is replaced by acidic amino acids and the transactivation domain is deleted, producing a molecule that forms stable heterodimers with the leucine zipper region of C/EBPs (35). Expression of A-C/EBP in a cell inhibits endogenous WT C/EBPβ by blocking its DNA binding activity and thereby interrupting its transcriptional function (35;36).

In HESCs treated with a "decidualization cocktail" containing P, E, and a cAMP analog, the expression of A-C/EBP in the transduced cells had no significant effect on the expression levels of either C/EBPβ or PR mRNA (Fig. 4.2C). In contrast, it strongly inhibited the expression of mRNAs corresponding to the well-known decidualization markers, PRL and IGFBP-1, indicating that the decidualization process is inhibited upon the expression of this dominant negative mutant. Our results also indicated that A-C/EBP

drastically reduced expressions of STAT3 mRNA. These results clearly demonstrated that the dominant-negative mutant repressed human decidualization by specifically blocking the transcriptional activity of the endogenous C/EBPβ. STAT3 is, therefore, one of the candidate transcriptional targets of C/EBPβ during this differentiation process.

Direct regulation of STAT3 promoter by C/EBPβ

To further investigate the mechanism by which C/EBPβ regulates STAT3 expression, we analyzed its interaction with the promoter of STAT3 gene. *In silico* analysis of the STAT3 promoter using the bioinformatics softwares TESS, TFSearch and Consite revealed that it contains four candidate C/EBPβ binding sites within the 2 kilo-base 5‘-flanking region of the gene at approximately -61, -521, -931 and -1470. To test whether C/EBPβ actually binds to one or more of these sites, we employed chromatin immunoprecipitation using an antibody specific for C/EBPβ. As shown in Fig. 4.3A, minimal binding of C/EBPβ was seen at the –61, -521, and –1470 sites. A relatively strong binding of this transcription factor was observed at the STAT3 promoter region -931.

To further confirm that this candidate binding region at –931, containing the CCAAT motif, is linked to the transcriptional status of the endogenous C/EBPβ, we employed the dominant negative mutant A-C/EBP, which inhibits the DNA binding ability of C/EBPβ. As shown in Fig. 4.2C, adenovirus-mediated expression of A-C/EBP strongly reduced the expression of STAT3 mRNA. When we examined the occupancy of the STAT3 promoter by C/EBPβ under these conditions, we found that A-C/EBP expression blocked the binding of C/EBPβ to the -931 region of STAT3 promoter (Fig. 4.3B). These data suggested that this region of the STAT3 promoter represents a major C/EBPβ binding site and likely mediates the direct transcriptional regulation of this cytokine signal transducer by C/EBPβ.

STAT3 controls human endometrial stromal decidualization

To investigate whether STAT3 plays a regulatory role in the stromal differentiation process, we employed siRNA strategy to reduce its expression and then

examined the effect of this intervention on decidualization by measuring the expression of classical biomarkers of this process. As shown in Fig. 4.4, siRNA-mediated attenuation of STAT3 mRNA expression (Fig. 4.4A) and consequent down-regulation of STAT3 protein (Fig. 4.4B) led to a marked reduction in the expression of differentiation markers, such as PRL and IGFBP-1, but did not significantly affect the mRNA level of C/EBPβ and PR (Fig. 4.4A). The reduction of PRL at the protein level was further confirmed by ELISA analysis (Fig. 4.4C). These results indicated that C/EBPβ-mediated STAT3 expression plays an essential role in human endometrial stromal decidualization.

Identification of STAT3-regulated gene networks that control HESC differentiation

Since STAT3 is regulated by C/EBPβ and controls the differentiation of human endometrial stromal cell in primary culture, we hypothesized that several C/EBPβ target genes will also be regulated by STAT3. To identify these STAT3-regulated genes during HESC decidualization, we suppressed its expression by a specific siRNA. The STAT3-deficient cells were then subjected to decidualization upon addition of the hormone cocktail. After 72 h of treatment, we collected total RNA from these cells and compared the gene expression profiles of control siRNA-treated and STAT3 siRNA treated HESCs. In this microarray analysis, we found that the expression of 759 genes was down regulated (>1.5 fold) in response to treatment with STAT3 siRNA. In contrast, the expression of 1046 genes was up regulated (>1.5 fold) in response to a similar treatment. Further analysis of these microarray-derived genes by Panther classification system revealed various molecular pathways that are controlled downstream of STAT3 signaling during the HESC decidualization process (Table 1).

A comparison of the gene expression profile regulated by STAT3 with that regulated by C/EBPβ, revealed common pathways that act downstream of both factors. Our analysis uncovered 336 genes whose expression was down regulated upon loss of C/EBPβ or STAT3 and 457 genes whose expression was up regulated upon loss of C/EBPβ or STAT3. The microarray-derived, which are controlled by both C/EBPβ and STAT3, are shown in Table 2.

SphK1 is a common downstream target of STAT3 and C/EBPβ during stromal differentiation

A prominent gene whose expression was strongly down regulated in C/EBPβ or STAT3-deficient HESCs was that encoding SphK1, a kinase that catalyzes the ATP-dependent phosphorylation of sphingosine to form sphingosine phosphate (S1P) (Table 2). To confirm the regulation of SphK1 by STAT3, we attenuated the expression of STAT3 mRNA by siRNA followed by treatment with hormone cocktail for three days. When the SphK1 mRNA expression was monitored, we found that it was indeed drastically reduced in the absence of STAT3 (Fig. 4.5A).

There are two major sphingosine kinases, SphK1 and SphK2 (37). Next we analyzed the temporal expression profiles of these two kinases in differentiating HESCs *in vitro*. Although the expression of SphK2 mRNA was detected in differentiating stromal cells, its expression level is much less compared to that of SphK1 (Fig. 4.5B). We noted that the expression of Sphk1 mRNA increased as the stromal cells differentiate, reached a peak on days 3 and 4, and gradually declined thereafter. The temporal expression pattern of SphK1 mRNA, therefore, overlapped closely with that of active STAT3 protein, consistent with our finding that SphK1 is a downstream target of STAT3.

We then investigated whether Sphk1 plays a role in HESC decidualization. We employed siRNA strategy to reduce SphK1 mRNA expression and then examined the effect of this intervention on decidualization by measuring the expression of classical biomarkers (PRL and IGFBP-1) of this process. As shown in Fig. 4.5C, the attenuation of SphK1 expression did not significantly affect the mRNA levels of PR and STAT3. In contrast, siRNA-mediated inhibition of SphK1 expression led to a marked reduction in the expression of PRL and IGFBP-1, as well as VEGF-A, a well-known angiogenic factor secreted by differentiating stromal cells.

To further ascertain that SphK1 controls HESC decidualization, we sought to reverse the SphK1 siRNA-mediated inhibition of decidualization by adding back the active product of the SphK1 enzyme. In this experiment, we treated the SphK1-defecient HESCs with S1P, which is a product of SphK1, to attempt to rescue the inhibition of

stromal decidualization. As shown in Fig. 4.5C, addition of S1P to SphK1 siRNA-treated stromal cells restored the expression of PRL, IGFBP-1 and VEGF-A mRNAs to levels comparable to that seen in control siRNA-treated cells, while this treatment did not significantly alter the expression levels of SphK1 and STAT3 mRNAs (Fig. 4.5C). Taken together, our results provided strong evidence that SphK1 is an important downstream target of STAT3 and a critical regulator of human endometrial stromal decidualization.

4.5 Discussion

C/EBPβ is long known as an acute phase protein in the liver and as a regulator of several genes involved in the regulation of inflammatory responses (38). A role for C/EBPβ as an inflammation regulator has not been explored in the context of a reproductive tissue. The present study revealed that C/EBPβ is a major regulator of signaling by the IL-11 cytokine, which is generally anti-inflammatory, during decidualization. IL-11 signals via a cell surface receptor complex comprising IL11 receptor α and the common signaling component gp130, and the signal is transmitted through the JAK/STAT pathway (39). A number of studies have shown that IL-11 signaling pathway is essential for decidualization. In mice, disruption of IL-11Rα expression leads to decidualization failure and infertility (15;31). In human endometrium, IL-11 and IL11Rα are expressed in decidualizing stromal cells of mid-late secretory-phase, suggesting a possible role in this process (22). In human primary stromal cultures, addition of IL-11 advanced P induced decidualization (34). *In vivo* immunostaining showed that this cytokine is significantly induced in the decidualizing stromal cells in the late secretory endometrium (22;40). In recent studies, dysregulation of IL-11-IL11Rα signaling pathway was linked to unexplained female infertility, presumably due to defects in decidualization (3;9;41). Our microarray analysis showed that IL11Rα and STAT3 mRNA expression are downstream of C/EBPβ, establishing an important functional link between this transcription factor and a local cytokine signal pathway, which is important for biological processes leading to the successful establishment of pregnancy.

Although locally produced cytokines facilitate decidualization, little is known about the underlying mechanisms by which these factors regulate differentiation (34;42).

The cytokines with identified roles in the female reprocuctive tract include the IL-6 family of cytokines, transforming growth factor beta superfamily, the interleukin 1(IL-1) family, and the colony-stimulting factors (CSFs) (43). Dysregulated expression or activation of these cytokines was reported to result in complete or partial failure of implantation, abnormal placental formation and recurrent miscarriage (6;7;44). Previous studies identified STAT3 as a major mediator of signaling by the IL-6 family of cytokines, which include IL-6, IL-11 and LIF (28). Our study indicated that primary HESCs produce ample amounts of IL-11 during decidualization (Fig. 4.1A), while only low amounts of IL-6 and LIF are synthesized in these cells (data not shown). Previous studies showed that STAT3 is a key signal transducer that is activated downstream of IL-11 signaling (45). It was reported that inhibition of STAT3 activation impairs implantation in the mouse (31). In our cell culture experiments, the temporal production of IL-11 by human stromal cells overlapped with the activation profile of STAT3 (Fig. 4. 1). Using various loss-of-function approaches, we showed that C/EBPβ regulates expression of STAT3. Using a siRNA strategy, we demonstrated that STAT3 expression critically controls human stromal decidualization. This conclusion was further strengthened via chromatin immunoprecipitation experiments, which revealed that C/EBPβ directly binds to the -931 region of the STAT3 promoter that contains a CCAAT motif. Expression of a dominant negative mutant A-C/EBP, which blocked DNA binding ability of endogenous C/EBPβ, also suppressed the STAT3 promoter occupancy by this transcription factor and as a consequence markedly reduced STAT3 mRNA production. Taken together, our studies provided convincing evidence that STAT3 is an essential mediator of C/EBPβ-driven human endometrial stromal differentiation.

As a transcriptional regulator, STAT3 is likely to regulate downstream gene pathways that help advance the differentiation process. STAT3 is also known to control angiogenesis and immunosuppression, biological processes intimately associated with decidual functions and embryo survival (23;46). To identify STAT3 target genes during stromal differentiation, we employed microarray analysis using HESCs in which STAT3 expression is attenuated by siRNA. We identified the gene encoding SphK1, a sphingosine kinase, as a STAT3-regulated gene. Interestingly, previous microarray

results indicated that SphK1 is also downstream of C/EBPβ, suggesting that a linear pathway involving C/EBPβ, STAT3 and SphK1 operates during HESC differentiation.

Sphingosines are lipids, which are implicated in diverse biological functions. The S1P and its receptors participate in a key signaling mechanism involved in angiogenesis (47), lymphocyte trafficking (48), proliferation, cell survival (37), and immunomodulaiton(49). Previous studies have shown that the sphingolipid pathway is activated in the decidua and potentially regulates uterine mesometrial angiogenesis during implantation (50). Mizugishi *et al* reported severely compromised fertility in SphK1-/-SphK2+/- female mice in which the production S1P is drastically reduced. The mutant phenotype was associated with impaired decidualization, infiltration of inflammatory cells, increased cell death in the decidua, and massive breakage of decidual blood vessels, leading to uterine hemorrhage and early pregnancy loss (51). These results underscored the importance of the sphingolipid signaling pathway in decidualization, maternal angiogenesis and immune-modulation during early pregnancy.

In the present study, we observed that SphK1 expression downstream of STAT3 critically controls human stromal decidualization. Loss of SphK1 expression results in impaired decidualization, which is reversed by addition of exogenous S1P. Furthermore, we found that SphK1 controls the expression of VEGF-A, a well-known mitogen for endothelial cells, thereby suggesting an important functional link between S1P signaling and angiogenesis in the human decidua. Therefore, our results in the in vitro decualization system are consistent with the findings in the SphK1-/-SphK2+/- female mice. It is likely that STAT3, acting directly or indirectly, regulates the expression of SphK1, which generates the important lipid metabolite S1P. This signaling molecule in turn acts via its receptor to control HESC differentiation. It is conceivable that during decidualization, the differentiating stromal cells produce and secrete critical paracrine factors, which promote endothelial cell proliferation and control immune cell recruitment, thereby regulating angiogenesis and maternal immune response during embryo implantation.

In summary, C/EBPβ-regulated expression of IL11Rα and STAT3 indicates that this signaling pathway plays a vital role during human endometrial stromal decidualization. We also found that the expression of SphK1, which generates key lipid metabolites, is downstream of both C/EBPβ and STAT3. Our study, therefore, uncovered a unique linear pathway involving C/EBPβ, IL-11Rα-STAT3 signaling, and sphingolipid signaling, which plays conserved and critical roles in regulating stromal differentiation, angiogenesis and immune response during decidualization.

4.6 Figures and legends

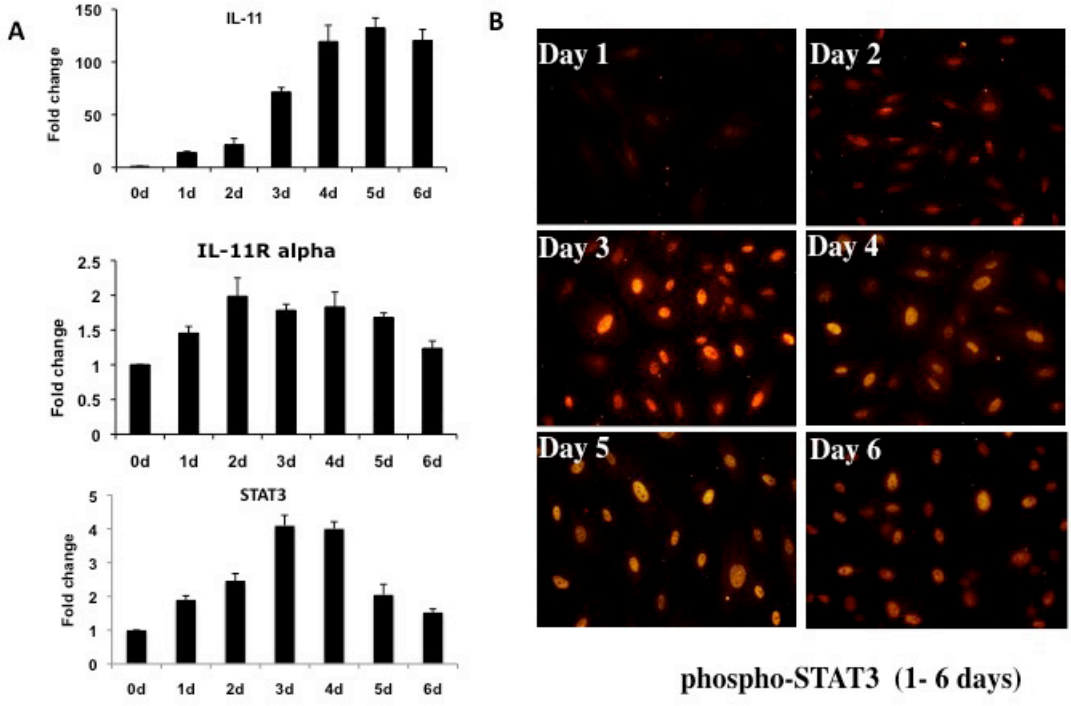

Figure 4.1 Activation of IL-11 signaling pathway during decidualization

HESCs were treated with hormone cocktail to induce decidualization *in vitro*.

A. Cells were harvested at different times during the decidualization process. Total RNA was isolated from these cells and real time PCR was performed to monitor the expression of IL11, IL11Rα and STAT3 mRNAs using gene-specific primers. Fold-changes were calculated with respect to the expression levels of these genes in stromal cultures prior to hormone cocktail addition (0 h). All real-time PCR data were normalized with respect to the mRNA level of 36B4.

B. The level of phospho-STAT3 (active phosphorylation at Ser 727) was monitored at different days of *in vitro* decidualization by immunocytochemistry using an antibody that specifically recognizes the Ser 727 phosphorylated form of STAT3.

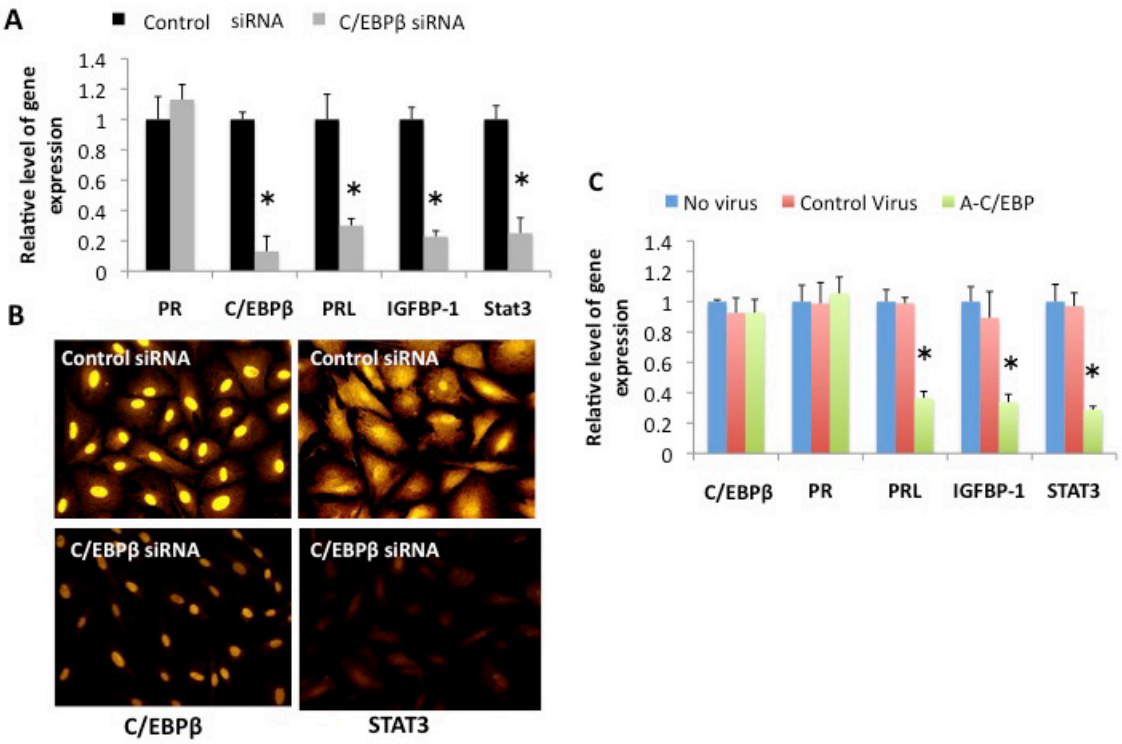

Figure 4.2 C/EBPβ controls the expression of STAT3 in differentiating stromal cells

HESCs were transfected with siRNA (40nM) targeted against C/EBPβ or control siRNA (scrambled). 24 h after transfection, HESCs were treated with hormone cocktail to initiate the stromal differentiation program *in vitro*.

A. Cells were harvested at 72 h following hormone treatment and total RNA was isolated. The expression of PR, C/EBPβ, PRL, IGFBP-1 and STAT3 mRNAs was measured by real-time PCR analysis using gene-specific primers. The expression of these genes in C/EBPβ siRNA treated cells (gray tower) is shown relative to that in control siRNA treated cells (black tower, set as 1.0). * indicates statistically significant change ($P<0.05$) when compared with control siRNA treatment.

B. The expression of C/EBPβ and STAT3 proteins was monitored after 72 h of hormone cocktail treatment by immunocytochemistry using antibodies that recognize C/EBPβ and STAT3, respectively.

Fig. 4.2 (continued) C/EBPβ controls the expression of STAT3 in differentiating stromal cells

C. HESCs were transduced with adenovirus expressing dominant negative mutant of C/EBP (A-C/EBP) or GFP (control)(MOI: 10:1). After 24 h of viral transduction, HESCs were treated with hormone cocktail for 72 h. RNA was isolated and the expression of C/EBPβ, PR, PRL, IGFBP-1 and STAT3 mRNA was analysis by real-time PCR using gene-specific primers. The mRNA levels were normalized with respect to that in untreated (no virus) control. * indicates statistically significant change ($P<0.05$) when compared with no adenovirus treatment.

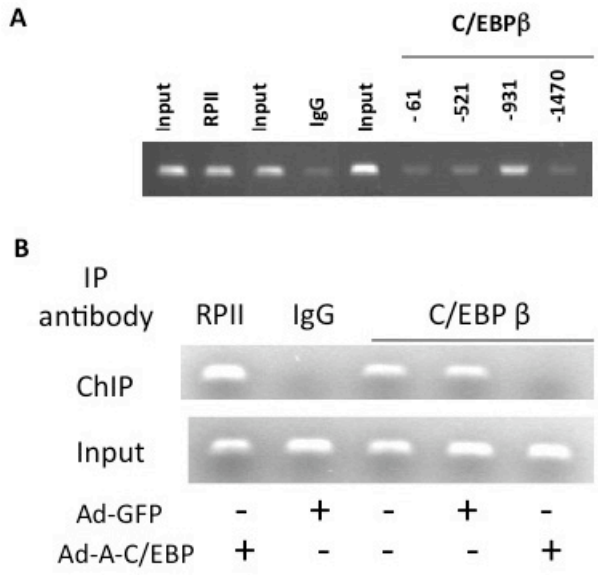

Figure 4.3 Regulation of STAT3 promoter by C/EBPβ in HESC

A, HESCs were treated with a hormonal cocktail containing E, P, and cAMP for 72 h. Cells were fixed with formaldehyde and ChIP assays were performed using anti-C/EBPβ, anti-RNA polymerase II and anti-rabbit IgG antibodies to immunoprecipitate the cross-linked DNA-protein complexes. After reversing the cross-links, DNAs isolated from the immunoprecipitates were used as templates for PCR using various primer sets to amplify specific regions of the STAT3 promoter. The nucleotide positions of the candidate C/EBPβ binding sites within the four promoter regions analyzed by ChIP are indicated. The experiment was repeated twice and representative data are shown.

B, HESCs were transduced with adenovirus expressing dominant negative mutant of C/EBPβ (A-C/EBP) or Ad-GFP-control (MOI: 10:1). 24 h after viral transduction, HESCs were treated with hormone cocktail for 72 h. Cells were treated with formaldehyde and ChIP assays were performed to determine promoter occupancy by C/EBPβ at the -931 site of the STAT3 promoter. For ChIP experiments, RNA Polymerase II (RPII) IP served as a positive control and IgG IP served as a negative control.

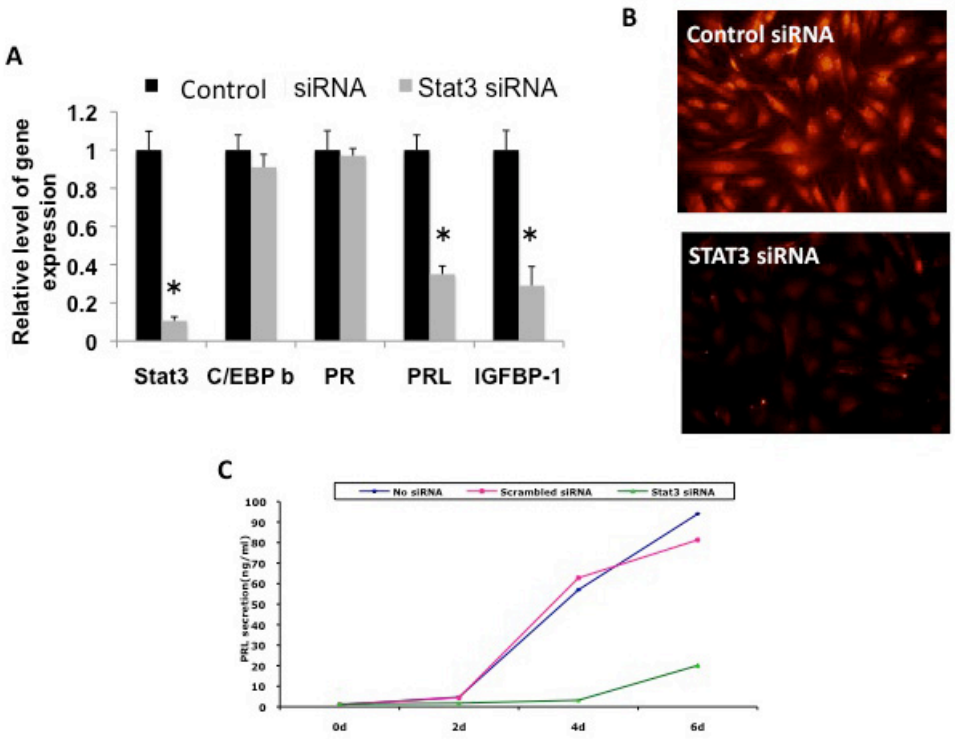

Figure 4.4 Suppression of STAT3 expression blocks HESC decidualization

HESCs were transfected with siRNA (20nM) targeted against STAT3 or control siRNA (scrambled). 24 h after transfection, HESCs were treated with hormone cocktail to initiate the stromal differentiation process *in vitro*.

A. Cells were harvested at 72 h following hormone treatment and total RNA was isolated. The expression of STAT3, C/EBPβ, PR, PRL and IGFBP-1 mRNAs was monitored by C/EBPβ siRNA treated cells (gray tower) is shown relative to that in control siRNA treated cells (black tower, set as 1.0). * indicates statistically significant change ($P<0.05$) when compared with control siRNA treatment.

B. The level of STAT3 protein was monitored after 72 h of hormone cocktail treatment by immunocytochemistry using an antibody that recognizes total STAT3.

C. Culture medium was collected at different time points after hormone cocktail treatment and the levels of secreted PRL were measured by an ELISA kit.

A: Down regulated pathways in HESCs treated with STAT3 siRNA

Pathway	REFLIST (n=25431)	ComLIST (n=749)	ComLIST (expected)	ComLIST (P value)
Wnt signaling pathway	348	22	10.25	1.45E-01
Inflammation mediated by chemokine and cytokine signaling pathway	315	19	9.28	5.22E-01
Angiogenesis	229	19	6.74	1.22E-02
Interleukin signaling pathway	194	18	5.71	4.64E-03
Integrin signalling pathway	227	18	6.69	3.23E-02
TGF-beta signaling pathway	154	15	4.54	1.24E-02
PDGF signaling pathway	189	15	5.57	1.06E-01
PI3 kinase pathway	121	13	3.56	1.42E-02
p53 pathway	136	11	4.01	4.62E-01
EGF receptor signaling pathway	150	11	4.42	9.46E-01
FGF signaling pathway	148	10	4.36	1.00E+00
T cell activation	111	9	3.27	1.00E+00
Endothelin signaling pathway	98	9	2.89	4.81E-01
Cadherin signaling pathway	168	8	4.95	1.00E+00
B cell activation	86	7	2.53	1.00E+00
VEGF signaling pathway	80	6	2.36	1.00E+00

B: Up regulated pathways in HESCs treated with STAT3 siRNA

Pathway	REFLIST (n=25431)	ComLIST (n=1032)	ComLIST (expected)	ComLIST (P value)
Inflammation mediated by chemokine and cytokine signaling pathway	315	33	12.81	2.41E-04
Wnt signaling pathway	348	27	14.15	2.32E-01
Angiogenesis	229	25	9.31	2.23E-03
PDGF signaling pathway	189	23	7.68	9.03E-04
Integrin signalling pathway	227	23	9.23	1.46E-02
p53 pathway	136	18	5.53	3.13E-03
FGF signaling pathway	148	14	6.02	6.00E-01
EGF receptor signaling pathway	150	14	6.1	6.73E-01
T cell activation	111	13	4.51	1.33E-01
Ras Pathway	91	13	3.7	2.08E-02
PI3 kinase pathway	121	13	4.92	2.83E-01
Interleukin signaling pathway	194	12	7.89	1.00E+00
Endothelin signaling pathway	98	12	3.98	1.43E-01
TGF-beta signaling pathway	154	11	6.26	1.00E+00
Cadherin signaling pathway	168	10	6.83	1.00E+00
VEGF signaling pathway	80	9	3.25	1.00E+00
Cytoskeletal regulation by Rho GTPase	111	9	4.51	1.00E+00
B cell activation	86	9	3.5	1.00E+00

Table 4.1 Pathways regulated by STAT3 during human endometrial stromal decidualization

Table 4.1 (continued) Pathways regulated by STAT3 during human endometrial stromal decidualization

A partial list of pathways, whose expression is down (A) and up (B) regulated (>1.5 fold change) in response to STAT3 siRNA treatment during HESC differentiation, is shown. The microarray data were analyzed using Panther software. REFLIST indicates the number of genes in NCBI H. sapiens database that are known to be involved in a particular pathway. ComLIST indicates the actual number of genes that are altered in this pathway upon siRNA treatment. ComLIST (expected) is the number of genes that are predicted to be affected by siRNA treatment. ComLIST (p-value) indicates the statistical analysis of the number of genes actually altered in the particular pathway.

A: Common down-regulated pathways in C/EBPβ and STAT3 Microarrays

Pathway	REFLIST (n=25431)	ComLIST (n=329)	ComLIST (expected)	ComLIST (P value)	Involved Genes
Interleukin signaling pathway	194	9	2.51	1.84E-01	FOXF1, FOXC1,IL15, STAT3, STAT5B,IL6R,IL6ST, MYC,
Wnt signaling pathway	348	8	4.5	1.00E+00	CDH11, TLE2,TLE3, MYC, SMARCD3, WNT2, WNT4, FAT4
Inflammation mediated by chemokine and cytokine signaling pathway	315	8	4.08	1.00E+00	STAT3,SOCS3, PLCL1,CCR1, CCL8, GRK5, MPP1, BCL3, SphK1
Angiogenesis	229	8	2.96	1.00E+00	STAT3, WNT2, VEGFA, PLD1, PIK3R1, SphK1,PTPN11, PDGFRA
TGF-beta signaling pathway	154	6	1.99	1.00E+00	BMP2, FOXC1, SMURF1, FOXF1,RHEB,SKI,
Integrin signalling pathway	227	6	2.94	1.00E+00	LAMA4, COL15A1,COL14A1, ABL1, PIK3R1, MAP3K5
PI3 kinase pathway	121	5	1.57	1.00E+00	FOXC1, FOXF1, INSR, PIK3R1, IGFBP1
PDGF signaling pathway	189	5	2.45	1.00E+00	STAT3, MYC, SHC3, STAT5B, PIK3R1,
EGF receptor signaling pathway	150	5	1.94	1.00E+00	STAT3,EREG,SHC3, STAT5B,MAP3K5,
Cadherin signaling pathway	168	5	2.17	1.00E+00	FAT4, LYN, CDH11, WNT2, WNT4
FGF signaling pathway	148	4	1.91	1.00E+00	FGF7, PTPN11, MAP3k5,SHC3,
VEGF signaling pathway	80	3	1.03	1.00E+00	VEGFA, SpHK1, PIK3R1

B: Common up-regulated pathways in C/EBPβ and STAT3 Microarray

Pathway	REFLIST (n=25431)	ComLIST (n=448)	ComLIST (expected)	ComLIST (P value)	Involved Genes
Wnt signaling pathway	348	16	6.13	9.53E-02	SFRP1,PPP2R5C, FZD7, PCDH7,CDH2,CDH6,FZD2, NLK, TBL1X, PLCB4, CCND3, CCND1, SMARCA2, PCDH9, TLE4, TCF7L2
Inflammation mediated by chemokine and cytokine signaling pathway	315	15	5.55	9.87E-02	PSCD2, SOCS6, AKT3, CXCR4, PRKX, ITGA1, ITGA4,GNAI2, PLCB4, RRAS, GRK6, RRAS,ITGA2,PLCB4,TNC,RGS4,C21
Integrin signalling pathway	227	14	4	1.14E-02	ITGA4,ITGB3,PIK3CD,COL11A1, COL8A1,COL4A6, RRAS, FLNA,RAP2B,ITGA3, CAV1, ITGA2, ITGA1,ACTN4
Angiogenesis	229	12	4.03	1.53E-01	SFRP1, PIK3R3, FZD2, CRYAS, RRAS, FLT1,TCF7L2,PDGFC, AKT3, LPXN, ANGPT2,
p53 pathway	136	8	2.4	5.32E-01	AKT3, THBS1, GADD45B, YWHAH, PIK3R3, YWHAE, NFATC2IP,SERPINE1,
PI3 kinase pathway	121	8	2.13	2.61E-01	AKT3, GNAI2, YWHAH, PIK3R3, YWHAE, CCND3, CCND1, RRAS,
FGF signaling pathway	148	8	2.61	8.74E-01	AKT3, PPP2R1B, PPP2R5C, SPRY1, YWHAH, YWHAE, SPRY4, RRAS,
EGF receptor signaling pathway	150	8	2.64	9.45E-01	AKT3, PPP2R5C, CBLB, SPRY1, YWHAH, YWHAE, SPRY4, RRAS,
TGF-beta signaling pathway	154	6	2.71	1.00E+00	TGFBR2, RRAS, INHBA, TGFB2, C21, SMURF2
PDGF signaling pathway	189	6	3.33	1.00E+00	AKT3, PRKX, ERF, PIK3R3, RRAS, DLC1,
Interleukin signaling pathway	194	6	3.42	1.00E+00	AKT3, IL6, IL23A, IL7, RRAS, C21,
VEGF signaling pathway	80	5	1.41	1.00E+00	AKT3, LPXN, PI3R3, RRAS, FLT1,
T cell activation	111	5	1.96	1.00E+00	CALM1, AKT3, PIK3R3, RRAS, CALM3,

Table 4.2 Pathways regulated by both C/EBPβ and STAT3 during human endometrial stromal decidualization

Table 4.2 (continued) Pathways regulated by both C/EBPβ and STAT3 during human endometrial stromal decidualization

A partial list of common genes, whose expression are down (A) and up (B) regulated (>1.5 fold change) by both C/EBPβ and STAT3 during HESC differentiation, is shown. The data were analyzed using Panther software. REFLIST indicates the number of genes in NCBI H. sapiens database that are known to be involved in a particular pathway. ComLIST indicates the actual number of genes that are altered in this pathway upon siRNA treatment. ComLIST (expected) is the number of genes that are predicted to be affected by siRNA treatment. ComLIST (p-value) indicates the statistical analysis of the number of genes actually altered in the particular pathway

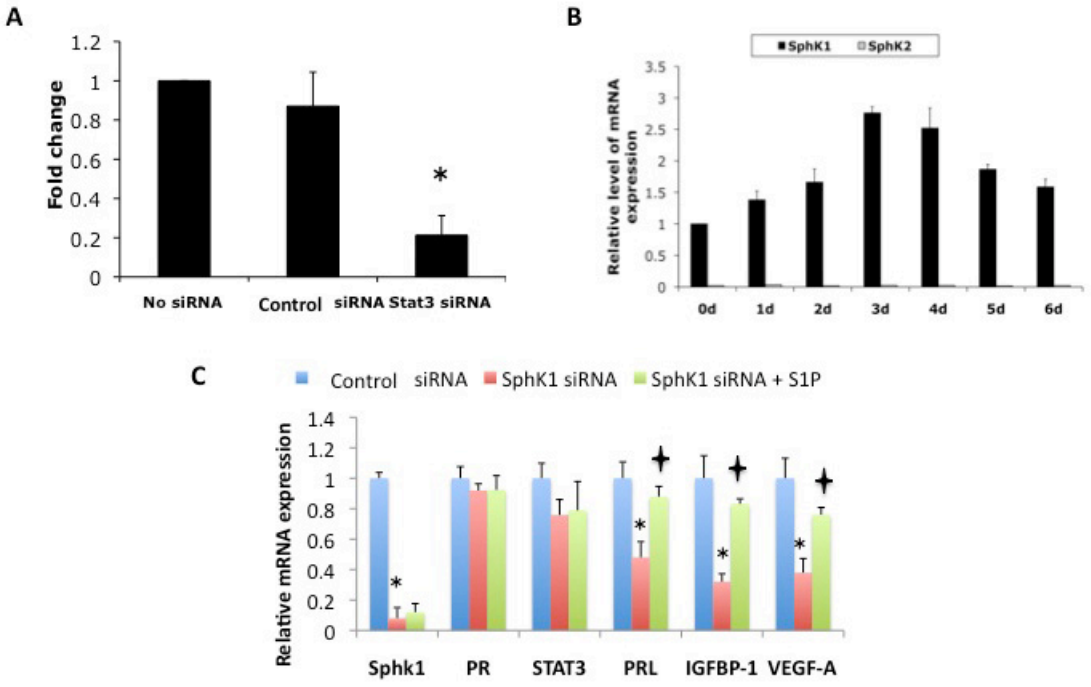

Figure 4.5 SphK1 is a target of STAT3 and controls HESC decidualization

A. STAT3 controls the expression of SphK1 mRNA during decidualization.

HESCs were transfected with siRNA (40nM) targeted against STAT3 or control siRNA (scrambled). After 24 h transfection, HESCs were treated with hormone cocktail for 72 h and the total RNA was isolated. The expression of SphK1 mRNA was monitored by real-time PCR analysis using gene-specific primers. Fold change was calculated with respect to the level of SphK1 mRNA in untreated cells. * indicates statistically significant change ($P<0.05$) when compared with no siRNA treatment.

B. Temporal expression of SphK1 and SphK2 mRNAs during HESC decidualization *in vitro*. HESCs were treated with hormone cocktail and harvested at different times during the decidualization process. Total RNA was isolated and real time PCR was performed to monitor the expression of SphK1 and SphK2 mRNAs using gene-specific primers. Fold-inductions were calculated with respect to the expression levels of these genes in stromal cultures prior to hormone cocktail addition (0 h).

C. Suppression of SphK1 mRNA expression by siRNA blocks HESC decidualization. HESCs were transfected with siRNA (40nM) against Sphk1 or control siRNA. 24h after

Fig. 4.5 (continued) SphK1 is a target of STAT3 and controls HESC decidualization

transfection, the hormone cocktail with or without S1P (5uM/ml) was added to the stromal culture. Total RNA was isolated after 72 h and the expression of SphK1, PR, STAT3, PRL, IGFBP-1 and VEGF-A were monitored by real time PCR using gene-specific primers. Fold change was calculated with respect to the expression of these genes in cells treated with control siRNA without S1P. *, $P<0.05$ compared with control siRNA treated cells. ✦, $P<0.05$ compared with SphK1 siRNA treated cells.

References

1. **Gellersen B, Brosens IA, Brosens JJ** 2007 Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives. Semin Reprod Med 25:445-453
2. **Maruyama T, Yoshimura Y** 2008 Molecular and cellular mechanisms for differentiation and regeneration of the uterine endometrium. Endocrine journal
3. **Karpovich N, Klemmt P, Hwang JH, McVeigh JE, Heath JK, Barlow DH, Mardon HJ** 2005 The production of interleukin-11 and decidualization are compromised in endometrial stromal cells derived from patients with infertility. J Clin Endocrinol Metab 90:1607-1612
4. **Chobotova K, Karpovich N, Carver J, Manek S, Gullick W, Barlow DH, Mardon HJ** 2005 Heparin-binding epidermal growth factor and its receptors mediate decidualization and potentiate survival of human endometrial stromal cells. J Clin Endocrinol Metab 90:913-919
5. **Salamonsen LA, Dimitriadis E, Jones RL, Nie G** 2003 Complex regulation of decidualization: a role for cytokines and proteases--a review. Placenta 24 Suppl A:S76-85
6. **Dimitriadis E, White CA, Jones RL, Salamonsen LA** 2005 Cytokines, chemokines and growth factors in endometrium related to implantation. Hum Reprod Update 11:613-630
7. **Laird SM, Tuckerman EM, Li TC** 2006 Cytokine expression in the endometrium of women with implantation failure and recurrent miscarriage. Reprod Biomed Online 13:13-23
8. **Wisniewski HG, Vilcek J** 2004 Cytokine-induced gene expression at the crossroads of innate immunity, inflammation and fertility: TSG-6 and PTX3/TSG-14. Cytokine Growth Factor Rev 15:129-146
9. **Dimitriadis E, Stoikos C, Stafford-Bell M, Clark I, Paiva P, Kovacs G, Salamonsen LA** 2006 Interleukin-11, IL-11 receptoralpha and leukemia inhibitory factor are dysregulated in endometrium of infertile women with endometriosis during the implantation window. J Reprod Immunol 69:53-64
10. **Klemmt P, Carver JG, Kennedy SH, Koninckx PR, Mardon HJ** 2006 Stromal cells from endometriotic lesions and endometrium from women with endometriosis have reduced decidualization capacity. Fertil Steril 85:564-572
11. **Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery CA, Jr., Shyamala G, Conneely OM, O'Malley BW** 1995 Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. Genes & development 9:2266-2278
12. **Gendron RL, Paradis H, Hsieh-Li HM, Lee DW, Potter SS, Markoff E** 1997 Abnormal uterine stromal and glandular function associated with maternal reproductive defects in Hoxa-11 null mice. Biology of reproduction 56:1097-1105

13. **Satokata I, Benson G, Maas R** 1995 Sexually dimorphic sterility phenotypes in Hoxa10-deficient mice. Nature 374:460-463

14. **Lim H, Paria BC, Das SK, Dinchuk JE, Langenbach R, Trzaskos JM, Dey SK** 1997 Multiple female reproductive failures in cyclooxygenase 2-deficient mice. Cell 91:197-208

15. **Robb L, Li R, Hartley L, Nandurkar HH, Koentgen F, Begley CG** 1998 Infertility in female mice lacking the receptor for interleukin 11 is due to a defective uterine response to implantation. Nature medicine 4:303-308

16. **Mantena SR, Kannan A, Cheon YP, Li Q, Johnson PF, Bagchi IC, Bagchi MK** 2006 C/EBPbeta is a critical mediator of steroid hormone-regulated cell proliferation and differentiation in the uterine epithelium and stroma. Proceedings of the National Academy of Sciences of the United States of America 103:1870-1875

17. **Lee K, Jeong J, Kwak I, Yu CT, Lanske B, Soegiarto DW, Toftgard R, Tsai MJ, Tsai S, Lydon JP, DeMayo FJ** 2006 Indian hedgehog is a major mediator of progesterone signaling in the mouse uterus. Nat Genet 38:1204-1209

18. **Mukherjee A, Soyal SM, Fernandez-Valdivia R, Gehin M, Chambon P, Demayo FJ, Lydon JP, O'Malley BW** 2006 Steroid receptor coactivator 2 is critical for progesterone-dependent uterine function and mammary morphogenesis in the mouse. Mol Cell Biol 26:6571-6583

19. **Gellersen B, Brosens J** 2003 Cyclic AMP and progesterone receptor cross-talk in human endometrium: a decidualizing affair. J Endocrinol 178:357-372

20. **Kim JJ, Buzzio OL, Li S, Lu Z** 2005 Role of FOXO1A in the regulation of insulin-like growth factor-binding protein-1 in human endometrial cells: interaction with progesterone receptor. Biology of reproduction 73:833-839

21. **Bilinski P, Roopenian D, Gossler A** 1998 Maternal IL-11Ralpha function is required for normal decidua and fetoplacental development in mice. Genes & development 12:2234-2243

22. **Dimitriadis E, Salamonsen LA, Robb L** 2000 Expression of interleukin-11 during the human menstrual cycle: coincidence with stromal cell decidualization and relationship to leukaemia inhibitory factor and prolactin. Molecular human reproduction 6:907-914

23. **Chen Z, Han ZC** 2008 STAT3: a critical transcription activator in angiogenesis. Medicinal research reviews 28:185-200

24. **McLoughlin RM, Jenkins BJ, Grail D, Williams AS, Fielding CA, Parker CR, Ernst M, Topley N, Jones SA** 2005 IL-6 trans-signaling via STAT3 directs T cell infiltration in acute inflammation. Proceedings of the National Academy of Sciences of the United States of America 102:9589-9594

25. **Wei LH, Kuo ML, Chen CA, Chou CH, Lai KB, Lee CN, Hsieh CY** 2003 Interleukin-6 promotes cervical tumor growth by VEGF-dependent angiogenesis via a STAT3 pathway. Oncogene 22:1517-1527

26. **Cheng JG, Chen JR, Hernandez L, Alvord WG, Stewart CL** 2001 Dual control of LIF expression and LIF receptor function regulate Stat3 activation at the onset of uterine receptivity and embryo implantation. Proceedings of the National Academy of Sciences of the United States of America 98:8680-8685

27. **Kortylewski M, Yu H** 2008 Role of Stat3 in suppressing anti-tumor immunity. Curr Opin Immunol 20:228-233

28. **Levy DE, Inghirami G** 2006 STAT3: a multifaceted oncogene. Proceedings of the National Academy of Sciences of the United States of America 103:10151-10152

29. **Niu G, Wright KL, Huang M, Song L, Haura E, Turkson J, Zhang S, Wang T, Sinibaldi D, Coppola D, Heller R, Ellis LM, Karras J, Bromberg J, Pardoll D, Jove R, Yu H** 2002 Constitutive Stat3 activity up-regulates VEGF expression and tumor angiogenesis. Oncogene 21:2000-2008

30. **Catalano RD, Johnson MH, Campbell EA, Charnock-Jones DS, Smith SK, Sharkey AM** 2005 Inhibition of Stat3 activation in the endometrium prevents implantation: a nonsteroidal approach to contraception. Proceedings of the National Academy of Sciences of the United States of America 102:8585-8590

31. **Nakamura H, Kimura T, Koyama S, Ogita K, Tsutsui T, Shimoya K, Taniguchi T, Koyama M, Kaneda Y, Murata Y** 2006 Mouse model of human infertility: transient and local inhibition of endometrial STAT-3 activation results in implantation failure. FEBS letters 580:2717-2722

32. **Teng CB, Diao HL, Ma XH, Xu LB, Yang ZM** 2004 Differential expression and activation of Stat3 during mouse embryo implantation and decidualization. Molecular reproduction and development 69:1-10

33. **Chen CL, Hsieh FC, Lieblein JC, Brown J, Chan C, Wallace JA, Cheng G, Hall BM, Lin J** 2007 Stat3 activation in human endometrial and cervical cancers. British journal of cancer 96:591-599

34. **Dimitriadis E, Robb L, Salamonsen LA** 2002 Interleukin 11 advances progesterone-induced decidualization of human endometrial stromal cells. Molecular human reproduction 8:636-643

35. **Greenwel P, Tanaka S, Penkov D, Zhang W, Olive M, Moll J, Vinson C, Di Liberto M, Ramirez F** 2000 Tumor necrosis factor alpha inhibits type I collagen synthesis through repressive CCAAT/enhancer-binding proteins. Mol Cell Biol 20:912-918

36. **Zhang JW, Tang QQ, Vinson C, Lane MD** 2004 Dominant-negative C/EBP disrupts mitotic clonal expansion and differentiation of 3T3-L1 preadipocytes. Proceedings of the National Academy of Sciences of the United States of America 101:43-47

37. **Spiegel S, Milstien S** 2003 Sphingosine-1-phosphate: an enigmatic signalling lipid. Nat Rev Mol Cell Biol 4:397-407

38. **Poli V** 1998 The role of C/EBP isoforms in the control of inflammatory and native immunity functions. The Journal of biological chemistry 273:29279-29282

39. **Heinrich PC, Behrmann I, Muller-Newen G, Schaper F, Graeve L** 1998 Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. Biochem J 334 (Pt 2):297-314

40. **Cork BA, Li TC, Warren MA, Laird SM** 2001 Interleukin-11 (IL-11) in human endometrium: expression throughout the menstrual cycle and the effects of cytokines on endometrial IL-11 production in vitro. J Reprod Immunol 50:3-17

41. **Dimitriadis E, Sharkey AM, Tan YL, Salamonsen LA, Sherwin JR** 2007 Immunolocalisation of phosphorylated STAT3, interleukin 11 and leukaemia inhibitory factor in endometrium of women with unexplained infertility during the implantation window. Reprod Biol Endocrinol 5:44

42. **Dimitriadis E, Stoikos C, Baca M, Fairlie WD, McCoubrie JE, Salamonsen LA** 2005 Relaxin and prostaglandin E(2) regulate interleukin 11 during human endometrial stromal cell decidualization. J Clin Endocrinol Metab 90:3458-3465

43. **Salamonsen LA, Hannan NJ, Dimitriadis E** 2007 Cytokines and chemokines during human embryo implantation: roles in implantation and early placentation. Semin Reprod Med 25:437-444

44. **van Mourik MS, Macklon NS, Heijnen CJ** 2009 Embryonic implantation: cytokines, adhesion molecules, and immune cells in establishing an implantation environment. J Leukoc Biol 85:4-19

45. **Dimitriadis E, Stoikos C, Tan YL, Salamonsen LA** 2006 Interleukin 11 signaling components signal transducer and activator of transcription 3 (STAT3) and suppressor of cytokine signaling 3 (SOCS3) regulate human endometrial stromal cell differentiation. Endocrinology 147:3809-3817

46. **Stepkowski SM, Chen W, Ross JA, Nagy ZS, Kirken RA** 2008 STAT3: an important regulator of multiple cytokine functions. Transplantation 85:1372-1377

47. **Waeber C, Blondeau N, Salomone S** 2004 Vascular sphingosine-1-phosphate S1P1 and S1P3 receptors. Drug News Perspect 17:365-382

48. **Rosen H, Sanna G, Alfonso C** 2003 Egress: a receptor-regulated step in lymphocyte trafficking. Immunol Rev 195:160-177

49. **Payne SG, Milstien S, Barbour SE, Spiegel S** 2004 Modulation of adaptive immune responses by sphingosine-1-phosphate. Semin Cell Dev Biol 15:521-527

50. **Skaznik-Wikiel ME, Kaneko-Tarui T, Kashiwagi A, Pru JK** 2006 Sphingosine-1-phosphate receptor expression and signaling correlate with uterine prostaglandin-endoperoxide synthase 2 expression and angiogenesis during early pregnancy. Biology of reproduction 74:569-576

51. **Mizugishi K, Li C, Olivera A, Bielawski J, Bielawska A, Deng CX, Proia RL** 2007 Maternal disturbance in activated sphingolipid metabolism causes pregnancy loss in mice. J Clin Invest 117:2993-3006

CHAPTER 5

CONCLUSION

During early pregnancy, the maternal steroid hormones estrogen and progesterone promote - decidualization, a process in which the uterine stromal cells undergo extensive proliferation and differentiation and are transformed into decidual cells. This physiological event is an essential prerequisite for embryo implantation and its disruption leads to dysfunctions in uterine receptivity, resulting in female infertility. However, the molecular mechanisms underlying decidualization are not fully understood. The overall objective of my thesis project was to explore the functional role of C/EBPβ, a transcription factor, as a unique regulator of decidualization. Using the C/EBPβ-null mouse model, we investigated the mechanisms by which C/EBPβ controls stromal proliferation during decidualization in the mouse and the human. We discovered that the deficiency of this transcription factor leads to a mitotic arrest of the stromal cells in mouse. Further studies showed that C/EBPβ controls the stromal cell cycle by governing the expression of several key regulatory molecules, including B-type cyclins, cdc25C, p53, p21 and p27, which play critical roles in the G2-M transition of the proliferating mouse uterine stromal cells. Interestingly, we found that in human endometrial stromal cells C/EBPβ controls the G1-S phase transition by regulating the expression of cyclin E and E2F1, which are critical regulators of DNA replication. These findings revealed that C/EBPβ controls uterine stromal proliferation in both mouse and human. It exerts this effect by regulating the expression of distinct cell cycle factors in a species-specific manner and, as a consequence, controls different stromal cell cycle stages in these species.

We employed an *in vitro* primary culture system to further establish that C/EBPβ controls the differentiation of human stromal cells. DNA microarray analyses identified the regulatory pathways downstream of this transcription factor that control the physiological events during decidualization (Fig. 5.1). It was uncovered that C/EBPβ

controls several key components of the cytokine signaling pathway, including the cytokine IL11, its receptor and signal transducer-STAT3. Additionally, C/EBPβ was noted to control the expression of BMP2 and Wnt4, two important regulators of human endometrial stromal differentiation.

The current challenge is to determine the mechanisms by which C/EBPβ controls events, such as angiogenesis and immunosuppression, during decidualization. Our recent studies suggest that C/EBPβ acts through STAT3 to control the expression VEGF-A and SphK1, which play key roles in regulating vascular permeability and angiogenesis during early pregnancy. Another potential function of C/EBPβ, channeled via activated STAT3, is to modulate the maternal immune response. Studies employing DNA microarray identified several paracrine factors downstream of STAT3 that might influence the conversion of uterine immune progenitor cells into distinct immune cells, reduce their cytotoxicity, and promote immuno-suppression to protect the implanted embryo. Further exploration of the downstream pathways of C/EBPβ in uterine stromal cells would help provide a better understanding of decidualization and implantation, and advance our knowledge of the causes underlying unexplained pregnancy loss, thereby improving fertility.

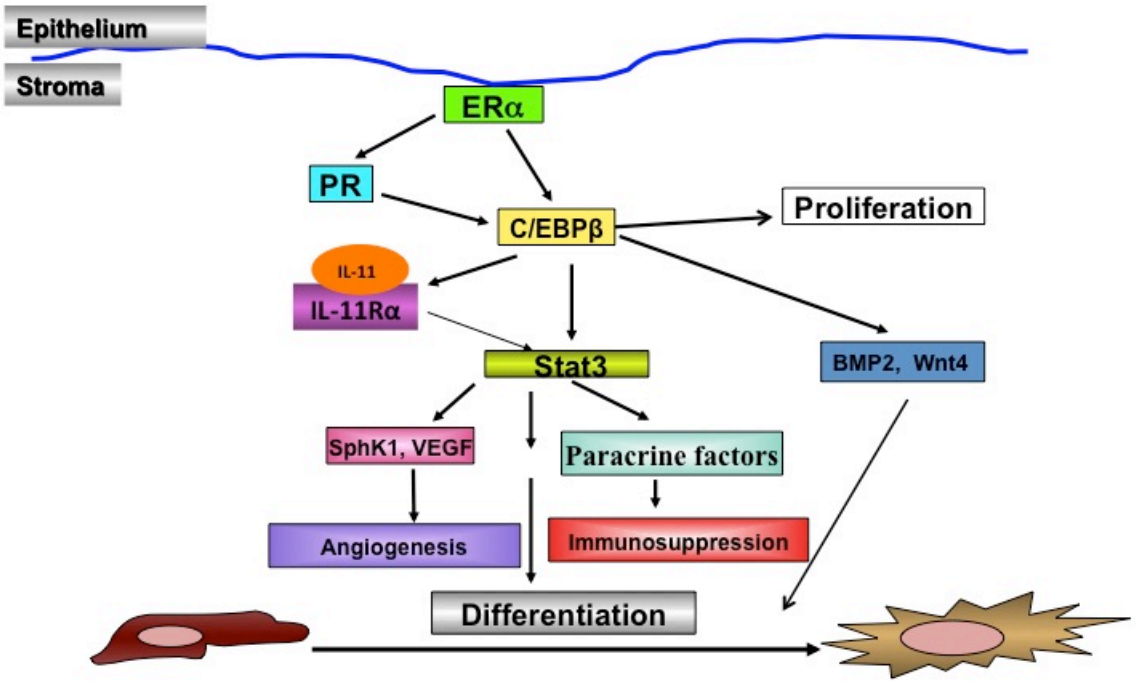

Figure 5.1 A schematic of the C/EBPβ-regulated pathways that control human endometrial stromal cell differentiation, angiogenesis, and immune response during decidualization.